ANALYSIS OF COMPONENTS RESPONSIBLE FOR THE CELLULAR LOCALIZATION AND ADHESIVE PROPERTIES OF CD44

by

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Date February 28, 2000
ABSTRACT

CD44 is a widely expressed cell adhesion molecule that binds the extracellular matrix component, hyaluronan in a tightly regulated manner. As a molecule involved in adhesion and cell migration, CD44 has been shown to interact with elements of the cytoskeleton via its cytoplasmic domain. A significant portion of CD44 is Triton X-100 insoluble in fibroblasts and this has been interpreted to reflect associations with the actin cytoskeleton. However, the detergent insolubility of CD44 in NIH 3T3 cells cannot be attributed to an interaction with the cytoskeleton since CD44 lacking a cytoplasmic tail remains insoluble after Triton X-100 extraction. Instead, after equilibrium density gradient centrifugation on sucrose, a proportion of CD44 was found in the low density lipid fractions, indicating that CD44 insolubility in NIH 3T3 cells is due to an association with Triton X-100-insoluble lipids. Furthermore, the migration of CD44 to the low density fraction is dependent on the transmembrane domain of CD44, not the cytoplasmic domain.

Previous studies have indicated that the CD44-hyaluronan interaction is affected by changes in the glycosylation state of CD44. Murine L cell variants defective in glycosaminoglycan synthesis and oligosaccharide processing were used to assess the effects of these modifications on CD44-mediated hyaluronan binding. Analysis of constitutive and antibody-induced hyaluronan binding ability in these cells indicated that sulfation, and in particular, modification by chondroitin sulfate is required for inducible hyaluronan binding in L cells. In the absence of fully processed oligosaccharides, chondroitin sulfate is not essential for hyaluronan binding, indicating that the effect of chondroitin sulfate is dependent on the glycosylation state of the cell. Thus, in addition to glycosylation, chondroitin sulfate biosynthesis is an important post-translational modification that can affect CD44-mediated hyaluronan binding.

CD44-hyaluronan interactions have been implicated in leukocyte rolling and extravasation at inflammatory sites. CD44 is normally expressed on leukocytes in an inactive state that cannot bind hyaluronan but can be converted to an active state upon activation by antigen or cytokines. Here it is demonstrated that the pro-inflammatory cytokine tumor necrosis factor α,
but not interferon γ, could convert CD44 from an inactive to an active hyaluronan binding form by inducing the sulfation of CD44 on the SR91 myeloid cell line. This post-translational modification was required for CD44-mediated binding to hyaluronan and to the vascular endothelial cell line, SVEC4-10. Tumour necrosis factor α-inducible hyaluronan binding was also observed on peripheral blood mononuclear cells, especially on CD14 positive monocytes and hyaluronan binding appeared to be at least partially sulfation dependent. In addition, monocyte-specific pro-inflammatory chemokines macrophage inflammatory protein-1α and monocyte chemoattractant protein-1 could induce hyaluronan binding in a subpopulation of peripheral blood mononuclear cells although it could not be determined definitively that the induction was sulfation dependent. Taken together, sulfation may be a means of regulating CD44-mediated adhesion, both in leukocytes at inflammatory sites as well as in fibroblasts to facilitate cell migration.
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LIST OF ABBREVIATIONS

APS  adenosine 5' phosphosulfate
ATP  adenosine triphosphate
bFGF  basic fibroblast growth factor
BFU-E  blast forming unit erythroid
BHK  baby hamster kidney cell
BSA  bovine serum albumin
C2GnT  core 2 β1-6 N-acetyl glucosaminyltransferase
CD44H  CD44 standard or haematopoietic isoform
CD44V  CD44 variant isoforms
CFU-GM  colony forming unit-granulocyte macrophage
CMFDA  5-chloromethyl fluorescein diacetate
CS  chondroitin sulfate
CSA  chondroitin 4-sulfate
CSC  chondroitin 6-sulfate
DABCO  2, 4 diazo bicyclo [2-2-2] octane
dD
t
DDM  2, 4 diazo bicyclo [2-2-2] octane
dH
t
DIG  detergent insoluble glycolipid enriched complex
DMEM  Dulbecco’s modified Eagle medium
DMSO  dimethylsulfoxide
DTH  delayed-type hypersensitivity
ECL  enhanced chemiluminescence
ECM  extracellular matrix
EDTA  ethylenediaminetetraacetic acid
endoH  endoglycosidase H
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<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ERM</td>
<td>ezrin, radixin, moesin</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FcR</td>
<td>Fc receptor</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FL-HA</td>
<td>fluoresceinated hyaluronan</td>
</tr>
<tr>
<td>FSC</td>
<td>forward scatter</td>
</tr>
<tr>
<td>G-actin</td>
<td>globular actin</td>
</tr>
<tr>
<td>G-CSF</td>
<td>granulocyte colony stimulating factor</td>
</tr>
<tr>
<td>GAG</td>
<td>glycosaminoglycan</td>
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<tr>
<td>Gal</td>
<td>galactose</td>
</tr>
<tr>
<td>GalNac</td>
<td>N-acetyl galactosamine</td>
</tr>
<tr>
<td>GEM</td>
<td>glycolipid enriched membrane fraction</td>
</tr>
<tr>
<td>GlcNac</td>
<td>N-acetyl glucosamine</td>
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<td>GlyCAM-1</td>
<td>glycosylation dependent cell adhesion molecule 1</td>
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<tr>
<td>GM-CSF</td>
<td>granulocyte-macrophage colony stimulating factor</td>
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<td>GPI</td>
<td>glycoposphatidyl inositol</td>
</tr>
<tr>
<td>HA</td>
<td>hyaluronan</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks’ balanced salt solution</td>
</tr>
<tr>
<td>HEV</td>
<td>high walled endothelial venule</td>
</tr>
<tr>
<td>HMEC</td>
<td>human microvascular endothelial cell</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>HS</td>
<td>heparan sulfate</td>
</tr>
<tr>
<td>HSV-1</td>
<td>herpes simplex virus 1</td>
</tr>
<tr>
<td>HUVEC</td>
<td>human umbilical vein endothelial cell</td>
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<tr>
<td>ICAM-1,2</td>
<td>intercellular cell adhesion molecule 1,2</td>
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IFNγ  interferon-γ
Ig   immunoglobulin
IL-1β interleukin-1β
IL-3  interleukin-3
IL-5  interleukin-5
IL-8  interleukin-8
IL-15 interleukin-15
kD   kilodalton
KS   keratan sulfate
LAT  linker for activation of T cells
LFA-1 lymphocyte function associated antigen
LPS  lipopolysaccharide
mAb  monoclonal antibody
MCP-1 monocyte chemoattractant protein-1
M-CSF macrophage colony stimulating factor
MDCK Madin-Darby canine kidney cell
MHC  major histocompatibility complex
MIP-1α macrophage inflammatory protein-1α
Mr   relative molecular mass
mRNA messenger ribonucleic acid
NMR  nuclear magnetic resonance
PAGE polyacrylamide gel electrophoresis
PAPS 3’ phosphate 5’ phosphosulfate
PBL  peripheral blood lymphocyte
PBMC peripheral blood mononuclear cell
PBS phosphate buffered saline
PC   phosphatidyl choline
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<tr>
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<td>PMA</td>
<td>phorbol myristate acetate</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PNA</td>
<td>peanut agglutinin</td>
</tr>
<tr>
<td>PNGaseF</td>
<td>peptide N-glycosidase F</td>
</tr>
<tr>
<td>PR</td>
<td>phenol red</td>
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<td>PSGL-1</td>
<td>P-selectin glycoprotein ligand-1</td>
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<tr>
<td>PVDF</td>
<td>polyvinylidene difluoride</td>
</tr>
<tr>
<td>RANTES</td>
<td>regulated upon activation, normally T cell expressed and secreted</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
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<tr>
<td>RT-PCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
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<tr>
<td>SCF</td>
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<tr>
<td>SDS</td>
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<td>TBS</td>
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<td>T cell receptor</td>
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<td>TCS</td>
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<tr>
<td>TRITC</td>
<td>tetramethyl rhodamine isothiocyanate</td>
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<tr>
<td>TSG-6</td>
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<td>very late antigen-4,5</td>
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I would like to thank all the members of the Johnson Lab, past and present, especially Mojgan Jabali, Ruihong Li and Kelly Brown (also known as the CD44 group) for useful discussions and for favours too numerous to mention. A special acknowledgement must go to my supervisor, Dr. Pauline Johnson, who has taught me to be meticulous and exacting both in the conception and execution of my work and who was willing to take a risk on a new idea which turned out to be very fruitful for all of us. Thanks go to Lesley Esford and Dr. Frank Tufaro, with respect to the collaborative work on GAGs and CD44 and Guitta Maki for teaching me all about the SR91 cells. I would also like to thank the members of my supervisory committee, Dr. Gerry Weeks, Dr. Wilf Jefferies, and Dr. Linda Matsuuchi, for all their guidance and help. I want to acknowledge the help of many members of the UBC community, the Teh, Levy, and Gold labs in the basement and others in the Dept. of Microbiology and Immunology, friendly lenders of reagents at the BRC and the Terry Fox Labs, Elaine Humphries at the EM facility and Dr. Rob Thies in the Dept. of Pharmaceutical Sciences. Personal thanks go to Ruihong and Kelly for help and moral support, Joan for stimulating and amusing discussions about science and life, and to Claire, Jacqueline, and Debbie for sanity saving dinners. Finally, I especially want to take this opportunity to thank Lesley for being a constant, supportive, and true friend.

I am not eloquent enough to find the appropriate words to express how much the love and support of my parents and sister has meant to me. I know my absence has been hard for them, as it has been for me, but I know that they have always respected and unconditionally supported all my decisions in life.
DEDICATION

To Mum and Dad
For everything

To Anne
Sister, and more importantly, friend

To Mr. F. Speed
For sparking in me, a passion for immunology one sunny, spring afternoon
(thank goodness I didn’t skip class for ice cream at Greg’s)
CHAPTER 1

Introduction

1.0 Leukocyte-endothelial cell interactions in the immune system

Lymphocyte “homing” allows the dispersal of the immunologic repertoire such that different lymphocyte subsets are directed to the appropriate microenvironments that support their survival and differentiation (reviewed in 1). This process is also at work during inflammatory responses when immune effector cells are targeted to sites of antigenic or microbial invasion. Most lymphocytes recirculate continuously from blood to tissue and back to the bloodstream, often twice per day (2). This recirculation is not a random event but is determined by lymphocyte-endothelial cell recognition and subsequent diapedesis of the lymphocyte across the vascular wall. The process of lymphocyte homing and homeostasis ensures that naive T and B lymphocytes recirculate between the blood and secondary lymphoid tissues (lymph nodes, Peyers patches, tonsils, and spleen) and requires lymphocyte binding to specialized endothelial cells called high walled endothelial venules (HEV), in the lymph nodes (3).

During inflammatory immune responses, neutrophils, monocytes, and activated T cells leave the bloodstream to enter tissues at a site of infection. The process of leukocyte migration (Fig. 1.1) has been divided into four separate and sequential steps: (i) “tethering” and “rolling”, transient and reversible primary adhesion that occurs within seconds and which is thought to be mediated by the selectin family of cell adhesion molecules; (ii) rapid leukocyte “activation” which occurs within seconds as chemokines activate integrin function; (iii) activation-dependent “arrest” that is stable under shear forces as integrins bind with high affinity to their receptors; (iv) diapedesis, or migration through the endothelial layer as cell adhesion molecules signal changes in the cytoskeleton in preparation for extravasation (reviewed in 1, 4, 5, 6). Different combinations of receptor-ligand interactions can occur at each of the first three steps leading to the selective behaviours of different leukocyte cell types in response to inflammatory agents.
Figure 1.1. Diagram of leukocyte adhesion and migration at an inflammatory site. Leukocytes are depicted in a capillary interacting with activated endothelial cells. The molecules implicated at each stage are indicated above the diagram. The bacterium in the tissue space represents the cause of the inflammatory response and the circles (○) represent the release of pro-inflammatory agents such as chemokines at the site of infection. HA is shown associated with activated endothelium and are depicted as chevrons.
During normal lymphocyte homing, carbohydrate-lectin interactions between the lymphocyte and the HEV retard the movement of lymphocytes. Here, selectin molecules play a role in lymphocyte rolling on HEV which then allows adhesion via integrins. Chemokines then upregulate integrin function, promoting firm adhesion followed by migration through the endothelial layer. A high rate of lymphocyte migration occurs through HEV because HEV endothelial cell junctions have a unique discontinuous flap-valve morphology, distinct from the typical occluding junctions formed by microvessel endothelia in extralymphoid tissues (3, 7). By contrast, upon initiation of an inflammatory response, neutrophils, monocytes, and effector T cells leave the circulatory system and enter the tissues. Inflammatory agents such as TNFα, IL-1, MCP-1, IL-8, and RANTES, to name a few, are released at the inflammatory site and act on the nearby microvascular endothelium and leukocytes to upregulate the function of various cell adhesion molecules (8-12). This results in increased expression or activation of the cell adhesion molecules ICAM-1, E- and P-selectin. These molecules then recruit activated leukocytes to the inflamed microvasculature by mediating leukocyte rolling, adhesion, arrest, and diapedesis. Some of the cell adhesion molecules involved in this process are outlined in Fig. 1.2. While similarities exist in the processes governing lymphocyte homing and leukocyte migration during inflammation, important differences must also exist as cells that extravasate to inflammatory sites are distinct from the naive T and B cell populations that bind to lymph node HEV. Implicit in this differential trafficking behaviour is the concept that activated/effector populations express a different array of cell adhesion molecules than naive cells. In addition, factors that act on leukocyte populations, such as cytokines and chemokines are localized to different microenvironments and different cell types express different combinations of receptors for these factors. Accordingly, the selective responses of different leukocytes to inflammatory agents as well as the recirculation patterns of lymphocytes can be explained by the combinatorial diversity in the molecular signals and leukocyte receptivity to distinct combinations of these signals. While some of the molecules involved at each step of the adhesion pathway have been identified, such as the selectins and the integrins, the molecules present at an inflammatory site are not well established and the role of CD44 in these
Figure 1.2. Molecules involved in leukocyte-endothelial cell interactions. The molecules implicated in leukocyte adhesion to endothelial cells in the lymph node or at inflammatory sites are illustrated. N-linked oligosaccharides are indicated by (●), O-linked oligosaccharides by (~~), and molecules that are known to be sulfated are indicated with a (★).
processes has not been well studied.

1.1 A role for CD44 and hyaluronan (HA) in leukocyte-endothelial cell interactions

Initial studies undertaken to identify a role for CD44 in leukocyte-endothelial cell interactions led to the generation of an anti-CD44 monoclonal antibody (mAb), Hermes-3. This mAb could block leukocyte adhesion to frozen mucosal lymph node sections (13). However, Hermes-3 did not block HA binding mediated by CD44 and other anti-CD44 mAbs were not able to block adhesion to the frozen sections in the murine or human systems (14). Furthermore, since the HA binding site of CD44 was an important factor in the binding of various cell lines and soluble CD44-Ig fusion proteins to endothelial cells (15-17), the discrepancies in the Hermes-3 data and subsequent identification of L-selectin in humans, diverted attention to the selectin molecules.

To support a role for CD44 in migration, a T lymphoma cell line activated to bind HA with phorbol myristate acetate (PMA) migrated to lymph nodes after subcutaneous injection into a BALB/c mouse (18). In a murine model of delayed-type hypersensitivity (DTH), the administration of anti-CD44 mAbs blocked leukocyte infiltration at a cutaneous site, but had no effect on lymphoid recirculation (19). This was indicative of a role, in vivo, for CD44 in the initial phases of the DTH response by facilitating extravasation into extralymphoid sites. Rolling adhesion mediated by CD44 and its ligand, HA has been demonstrated. In vitro studies looking at leukocytes under flow conditions have identified an interaction between CD44 on activated lymphocytes, and T and B cell lines, with HA on the endothelial cell line SVEC4-10 (20). CD44 has also been implicated in adhesion under flow between tonsillar lymphocytes or SKW3 T lymphoma cells and human tonsillar stromal cells (21). However, these cells could not interact with TNFα stimulated human umbilical vein endothelial cells (HUVEC). In both of these studies, lymphocyte rolling could be blocked by anti-CD44 mAbs or exogenously added HA. Further experiments have indicated that CD44 binding to HA could be transiently induced in T cells upon
superantigen stimulation in vivo. These cells exhibited CD44-HA dependent rolling and CD44-HA dependent extravasation at an inflammatory site (22, 23). This is the strongest evidence to date, for a role for CD44 and HA in T cell extravasation at inflammatory sites.

Although the role of selectins and integrins is well established in leukocyte rolling and adhesion, a role for CD44 is not as well characterized. Leukocyte extravasation to inflammatory sites is thought to occur primarily in the microvasculature and endothelial cells lining these vessels can upregulate HA production in response to pro-inflammatory factors in vitro. Many of these pro-inflammatory cytokines can induce the HA binding ability of CD44 on monocytes, which are known to migrate at these sites. Antigen induced T cell activation can transiently induce the HA binding ability of CD44, which would allow these cells to bind HA expressed on activated endothelial cells. Inhibition of T cell extravasation by treatment with anti-CD44 mAbs or injection of HA in vivo supports this idea (23). These data are consistent with a role for CD44 and HA in inducible adhesion at inflammatory sites, but not lymphocyte homing and the binding of naive lymphocytes to lymph node HEV. However, arguing against an important role for CD44 in inflammation is data from the CD44 gene knockout mice which report no significant change in the inflammatory response in the skin after induction of a DTH response (24).

1.2 CD44 expression and HA binding ability of leukocytes and endothelial cells

Resting leukocytes express CD44 but do not normally bind HA. However, lymphocytes, either isolated T cells or T cell clones, can be induced to bind HA after antigen, superantigen, or mitogen induced activation in vitro (22, 23, 25) and alloantigen or superantigen stimulation in vivo (23, 26, 27). This was demonstrated by leukocyte rolling on an immobilized HA substratum or by adhesion to HA. HA binding was often observed in a subpopulation of activated cells and was transient. For example, after in vivo alloantigen stimulation, the percentage of HA binding spleen cells was maximal at day 7 and returned to zero by day 12 (27). B cell lines can also roll on immobilized HA (20), and in vitro stimulation of B cells with IL-5 induced a subpopulation of cells to bind HA (28). Thus lymphocyte activation appears to induce a subpopulation of cells to bind
HA, although binding is transient. Monocyte activation can result in the induction of HA binding by CD44 as exposure of peripheral blood monocytes to inflammatory cytokines such as TNFα and IL-1 induced HA binding in a population of cells in a CD44-dependent manner (29, 30). Similarly, immature dendritic cells can be induced to mature and bind HA upon culture in TNFα (31).

Analysis of cultured endothelial cells derived from large vessels such as HUVEC cells or from the microvasculature such as human dermal microvascular endothelial cells (HMEC) demonstrates that these cells express CD44 but are unable to bind HA. Treatment of these cells with pro-inflammatory cytokines such as TNFα resulted in the upregulation of CD44 expression. However, only endothelial cells derived from the microvasculature were induced to bind HA (32, 33). Lymph node HEV and the SV40 transformed murine lymph node endothelial cell line, SVEC4-10, expressed surface bound HA (16), and this expression was elevated upon treatment with TNFα, IL-1β or lipopolysaccharide (LPS) for 4 hours (33). Increased surface expression of HA was not due to changes in mRNA levels of the HA synthetase or HA degrading enzymes (33). Histologically, HA was shown to be present in the intercellular spaces of vascular endothelial cells in skin capillaries (34).

HUVEC, which do not bind HA, only express the standard form of CD44, CD44H and none of the variant isoforms (CD44v). However, in another study, CD44H and CD44v3 containing isoforms were detected, although HA binding was not assessed (35). Analysis of wound microvascular endothelial cells revealed the presence of chondroitin sulfate (CS)-modified CD44H and CD44v3 (36), suggesting that there may be a correlation between CD44v isoform expression or CS modification and HA binding.

1.3 CD44 structure

CD44 is a cell adhesion molecule expressed on several cell types including haematopoietic cells, fibroblasts, many epithelial cells, and some endothelial cells and neural cells. This type I transmembrane glycoprotein is synthesized as a 37 kD core protein that is modified by
carbohydrate attachment to give rise to the most prevalent form of CD44, CD44H or CD44s, with a \( M_r \) of 85 000-90 000. CD44H represents the major form of CD44 on leukocytes and fibroblasts. Further modification of CD44H with chondroitin sulfate (CS) gives rise to a protein of 180-200 kD (37). The cloning of CD44 predicted a protein with a large extracellular domain, a 72 amino acid cytoplasmic domain, and a single transmembrane region of 21 amino acids (38-41). Alternative splicing of at least 10 variant exons (v1-10) of CD44 gives rise to multiple isoforms of CD44 (CD44v) which add sequence to the membrane proximal region of the extracellular domain (42). While these variant CD44v isoforms are less abundant than CD44H, they can be found expressed on the surface of epithelial cells, endothelial cells, activated lymphocytes, and some tumour cells.

The N-terminal of the extracellular domain of CD44 (amino acids 21-182) has ~85% sequence identity among mammalian species and contains a region with ~30% sequence identity to the Link module, a protein domain present in extracellular proteins that bind HA (38, 39). This region also contains six cysteine residues that are thought to be disulfide bonded. Determination of the structure of one of these Link module containing HA binding proteins, TSG-6, revealed a structure very similar to the C-type lectins (43). C-type lectin domains are found in the N-terminal region of the selectins, which play a role in the initial attachment of leukocytes to HEV or activated endothelium (4, 44, 45). Mutagenesis studies have implicated the N-terminal region of CD44 in binding to HA (46). Two clusters of basic amino acids were shown to mediate binding of CD44-immunoglobulin (CD44-Ig) fusion proteins to immobilized HA. Mutation of the more N-terminal cluster had severe consequences on HA binding with a requirement for arginine at position 41 (arg 41) for HA binding. Thus, a motif was proposed, B(X7)B (47), which was sufficient for HA binding (see Fig. 1.3 for details of CD44 structure). Mutational analysis of the HA binding site of CD44 based on the NMR structure of TSG-6 demonstrated residues in the exposed area close to arg 41 were critical for HA binding (48). Residues important for HA binding are thought to form a coherent surface on the Link module of CD44, running along a ridge in the protein surface. The Link homology region of CD44 contains the majority of N-linked glycosylation sites and
Figure 1.3. Schematic diagram of the CD44 molecule. BX,B is a motif known to play a role in HA binding. B represents a basic amino acid and X is a neutral or basic amino acid (47). The amino acids are numbered according to the human CD44H sequence (39). The Link homology domain and HA binding region are as indicated and the shaded region from amino acids 183 to 268 represents the membrane proximal region. Potential disulfide bonds are indicated as (S-S), N-linked oligosaccharides as (▼), potential O-linked oligosaccharides as (●), and (+++) represents GAG addition at the two conserved sites. The large arrow indicates the point of insertion for additional sequence which generate the CD44v isoforms. Three regions are indicated in the cytoplasmic domain. Amino acids 298-300 (KKK) has been implicated in ERM protein binding, amino acids 304-318 has been implicated in ankyrin binding, and the amino acids 331-332 (LV) has been implicated in basolateral sorting. Potential sites of serine phosphorylation are indicated with a P. From (49).
modification of these sites has been shown to affect the ability of CD44 to bind HA (reviewed in 50, 51).

The membrane proximal region of the CD44 extracellular domain (amino acids 183-268) is much less conserved between species (~35% identity) and contains multiple sites for O-linked glycosylation, two conserved serine-glycine-serine-glycine (SGSG) motifs for glycosaminoglycan (GAG) addition and the site of insertion of the alternatively spliced exons. The inclusion of additional sequence from the variant exons, as well as GAG addition and O-linked glycosylation can affect the HA binding ability of CD44 (reviewed in 50, 51, 52).

The transmembrane region of CD44 can influence HA binding by promoting the self-association of CD44 (53, 54). Various cytoplasmic domain deletions of CD44 expressed in T cell lines or transfected into COS cells can also reduce or abolish HA binding (15, 55-58), and prevent the migration of transfected melanoma cells on HA (59). The cytoplasmic tail is also responsible for localization of CD44 within the cell. In confluent, polarized epithelial cell cultures, CD44 is excluded from the apical region and localizes in the basolateral surface of the cells (60). This localization is mediated by a dipeptide leucine-valine (LV) in the cytoplasmic domain (61).

1.4 CD44 function

CD44 has been implicated in embryogenesis, lymphopoiesis and myelopoiesis, lymphocyte activation, progenitor homing, angiogenesis, wound healing, leukocyte rolling and extravasation at inflammatory sites, and tumour metastasis. Its precise role in these events is unclear as CD44 gene knockout mice appeared normal, showing no obvious developmental or functional abnormalities. Analysis of immune cell development and function did not reveal any significant defects although egress of granulocyte/monocyte progenitors from the bone marrow was impaired (24). These mice also developed exaggerated granuloma responses to Cryptosporidium parvum infection and tumor studies showed that SV40-transformed CD44-deficient fibroblasts were highly tumorigenic in nude mice, whereas re-introduction of CD44H expression into these fibroblasts resulted in a dramatic inhibition of tumor growth. Perhaps, CD44 has a redundant role and its function can be
compensated for by other cell adhesion molecules in the CD44 gene knockout mouse. The function of CD44 in the above-mentioned processes probably relates to its ability to participate in cell-cell adhesion and cell-extracellular matrix (ECM) interactions. In many cases, these associations are mediated by an interaction between CD44 and HA (14, 16, 62, 63). The importance of CD44-HA interactions can be inferred from studies of tumour cell growth and metastasis where dysregulation of these interactions occurs (reviewed in 52, 64, 65). In addition to mediating cell adhesion, CD44 has been implicated in cell migration. CD44 can signal the migration of melanoma and fibroblast cells, upon HA binding, during wound repair and appears to require serine phosphorylation of the cytoplasmic domain (59, 66, 67). CD44 can also mediate microvascular endothelial cell migration on fibrinogen during wound repair (36, 68) and vascular smooth muscle cell proliferation and migration in response to arterial wall injury (69). CD44-HA interactions and CD44 migration have also been implicated in a number of disease processes involving chronic inflammation such as murine arthritis (70, 71), inflammatory bowel disease and experimentally induced colitis (72), and in experimental autoimmune encephalomyelitis, a murine model of multiple sclerosis (73). However, some aspects of CD44 function, such as its involvement in migration of progenitor T cells to the thymus, may not involve an interaction with HA (74, 75).

1.5 CD44 ligands

CD44 is presently the most extensively characterized cell surface HA receptor and HA is the most extensively characterized CD44 ligand. Other molecules such as serglycin (76, 77) and the CS-modified invariant chain (78), have been identified as ligands that bind to CD44 via their CS side chains. Certain isoforms of CD44 (CD44v4-7, CD44v4-10, and CD44v10) can bind to chondroitin 4-sulfate (CSA) through the HA binding site of CD44 (79-81). Osteopontin, a cytokine-like molecule with adhesive and migratory functions, can bind to integrins in a calcium-dependent manner and to specific CD44v isoforms in a calcium-independent manner (82). CD44v isoforms may cooperate with β1 integrins to bind to osteopontin (83). CS modified forms of
CD44 have been reported to bind other β1 integrin ligands such as fibronectin (84), collagen III (85), and collagen XIV (86). CD44 can also mediate melanoma cell migration and invasion on type I and type IV collagen (87, 88). Modification of CD44 by the GAGs, CS, and heparan sulfate (HS) can augment the functions of CD44 by providing additional binding sites for chemokines such as MIP1-β (89), proteinases such as matrix metalloproteinase 9 (90), and heparin binding growth factors such as basic fibroblast growth factor (bFGF) and scatter factor (91, 92). These factors may themselves also play a role in cell adhesion, migration and invasion. By the construction of artificial proteoglycans, the specificity of different GAG modified versions of CD44 was revealed. Apparently, growth factors such as bFGF bind to HS-modified CD44 whereas chemokines such as RANTES (regulated upon activation, normally T cell expressed and secreted) bind to both HS-modified CD44 and CS-modified CD44 (93).

Hyaluronan (HA) is a high molecular weight GAG made up of repeating disaccharide units comprised of β(1-4)-D-glucuronic acid-β-(1-3)-N-acetyl-D-glucosamine (Fig. 1.4). HA is not sulfated or attached covalently to membrane proteins, unlike all other GAGs. HA is found in almost all vertebrate tissues as a component of the ECM and is present in large amounts in cartilage and synovial fluids. Evidence is gathering to suggest that HA can have a profound influence on cell behaviour. Levels of HA in the ECM are strictly regulated by cellular hyaluronidases and receptor-mediated endocytosis of HA. The ECM becomes enriched in HA coincident with periods of rapid cell proliferation, aggregation and migration during embryogenesis, and during tumour cell invasion (reviewed in 94, 95). HA expression is also transiently increased during DTH reactions in the skin (96). These effects on HA expression by differing cell types are, in turn, supported and directed by cell surface hyaladherins, such as CD44 (97).

1.6 CD44-HA mediated cell adhesion

CD44-HA mediated adhesion has been observed between a variety of cell types including a T cell line and an endothelial cell line (15); PMA activated T cells and human gingival fibroblasts (98); a B cell hybridoma and a bone marrow stromal cell line (62); and haematopoietic progenitors
Figure 1.4. Structure of the repeating disaccharide of hyaluronan. Hyaluronan is composed of repeating units of N-acetyl glucosamine and glucuronic acid. The number of repeating disaccharide units can range from 1 to over 1000. Adapted from (49).
have been shown to bind HA (99). Additionally, several examples of leukocytes and leukocyte cell lines binding to endothelial cells under static or flow conditions have been documented as described in previous sections. In all of these examples, HA is thought to act as a bridge mediating CD44-CD44 dependent adhesion. This CD44-HA-CD44 interaction is thought to occur by the presentation of surface bound HA by one cell (anchored by cell surface CD44) to CD44 on the surface of the opposing cell.

1.7 Regulation of the HA binding ability of CD44

The ability of CD44 to bind HA is tightly regulated and different cell types and cell lines exhibit different HA binding capacities (63). Based on the HA binding ability of CD44, cells can be classified into three groups: (i) cells expressing CD44 in an active state that bind HA constitutively; (ii) cells expressing CD44 in an inducible state where conversion to an active form from an inactive form occurs with appropriate stimuli; (iii) and cells expressing CD44 in an inactive state that cannot be induced to bind HA even in the presence of an inducing antibody. Although many cell lines bind HA constitutively, normal resting leukocytes usually do not (100). Conversion from an inactive form of CD44 to an active form capable of binding HA occurs in cells in response to stimuli such as PMA (101), an inducing antibody, or antigen (27). While many factors have been shown to affect the HA binding ability of CD44, the biochemical changes occurring in response to physiological stimuli have not been clearly delineated. Numerous studies have indicated that the expression of CD44v isoforms, associations with other proteins, post-translational modifications in the CD44 extracellular domain, the transmembrane domain, and the cytoplasmic domain are among the factors that can influence HA binding (reviewed in 50, 52). Given the surfeit of factors that can affect the HA binding status of CD44, two mechanisms by which these factors regulate HA binding are proposed: (1) by affecting the aggregation state of CD44 or; (2) by altering the conformation of CD44. These two mechanisms are not necessarily mutually exclusive.
The presence of CD44v isoforms can affect HA binding (102-105). The CD44v8-10 isoform (CD44R1) is able to bind HA constitutively (106, 107), although the same isoform differing in three amino acids (CD44E) is not (17). These contradictory data may reflect the fact that these isoforms were transfected into differing cell types (17, 102, 103, 107) and may reflect differences in amino acid sequence or the presence of cell-type specific regulatory factors in the transfected cells. Some CD44v isoforms (CD44v4-7) facilitate CD44 aggregation in the membrane and can induce HA binding (108). The fact that some CD44v isoforms can bind CS (79-81), suggests that GAG modification of CD44 may enhance CS-CD44/CD44v interactions, promoting aggregation.

Recently, interest has focused on understanding how the post-translational modification of CD44 influences its HA binding function. It has been demonstrated that removal of keratan sulfate (KS) from CD44H enhances HA binding (109) while the mutation of 4 SG motifs, potential sites of GAG addition, impairs HA binding by CD44 (110), suggesting that GAGs are regulators of CD44 function. Carbohydrate modification can also influence the ability of CD44 to bind HA (reviewed in 50, 51). O-linked carbohydrate addition occurs in the membrane proximal region of the CD44 extracellular domain. Some laboratories have reported that O-linked modification of CD44 is a negative regulatory factor with respect to HA binding (111-113) while others show no effect on HA binding using inhibitors of O-linked carbohydrate addition (114). However, data also showed that O-linked oligosaccharides, in particular N-acetyl galactosamines (GalNac) on CD44 expressed on cells enhanced its ability to bind HA but this was not observed using CD44-Ig fusion proteins, supporting the premise that O-linked glycosylation may affect the aggregation state of CD44 in the cell membrane (115). N-linked oligosaccharide addition occurs primarily within the CD44 HA binding domain. Generally, N-linked sugars have a negative effect on CD44-mediated HA binding (116, 117). One group has demonstrated that N-linked glycosylation is required for HA binding by CD44 in melanoma cells by mutagenesis of the 5 potential sites for N-linked carbohydrate addition (110). By contrast, others have shown that removal of carbohydrate from the first and fifth asparagine residues by mutagenesis can enhance HA binding (118). The
contradictory data regarding positive versus negative regulation of HA binding by the addition of N-linked oligosaccharides may reflect differences in the cell lines studied, although factors responsible for these differences have not been identified. Most of the evidence is in agreement regarding the negative influence of terminal sialic acids on CD44-mediated HA binding (113, 115, 116, 118), especially the presence of α2,3 sialic acids (115). Finally, it is evident that truncated N-linked carbohydrates can enhance HA binding and, in particular, the presence of the first N-linked N-acetyl glucosamine (GlcNac) residue is sufficient to promote HA binding (115). Changes to N-linked glycosylation, which occur primarily in the HA binding domain, may be predicted to act by altering the conformation or the accessibility of the CD44-HA binding domain.

The cytoplasmic domain of CD44 has been shown to be required for optimal HA binding and suggests that molecules associating with the cytoplasmic domain regulate CD44 function (55, 100). Cytoskeletal interactions with the cytoplasmic domain have been reported to enhance HA binding, perhaps by promoting the aggregation of CD44 on the cell surface (58, 119, 120). Serine phosphorylation of the cytoplasmic domain may also influence CD44 adhesion (121) although others found it was not required for HA binding (122). The requirement for multivalent antibody in order to induce HA binding by the anti-CD44 mAb IRAWB 14 suggests that aggregation and dimerization of CD44 are important (15, 123). HA binding can be enhanced by the self-association of CD44H via its transmembrane region (53, 54) and by artificial dimerization (55).

1.8 Regulation of HA binding in leukocytes

In most cells, a threshold level of CD44 expression is required before HA binding is observed (55). However, increases in CD44 expression alone are insufficient to induce HA binding and some post-translational modifications on CD44 have been implicated as a mechanism to induce HA binding. Antigenic stimulation induced the transient expression of CD44v6 isoforms in T cells, B cells, and macrophages (124) and CD44v isoforms, including CD44v6 were upregulated after antigen and mitogen activation of T cells (125). Antigen induced T cell activation increased CD44 expression and transiently increased HA binding in a subpopulation of T cells.
However, after *in vivo* allogeneic stimulation, no evidence of CD44v isoform expression was found in splenic cells (27). Leukocyte activation may also result in glycosylation changes on CD44. Culture of B cells in IL-5 or monocytes in TNFα resulted in the induction of HA binding and correlated with a decrease in N-linked carbohydrate on CD44 (126, 127). Differences in N-linked glycosylation and phosphorylation of CD44 have also been observed between resident (resting) and elicited (activated) macrophages, although HA binding was not examined and therefore could not be correlated with differences (128). However, in another example, no gross changes in the $M_t$ of CD44 was observed after superantigen activation of lymph node T cells, suggesting no major changes in the glycosylation state of CD44 (22).

### 1.9 Sulfation as a post-translational modification that regulates cell adhesion

Increasingly, glycoconjugates containing sulfate are being identified in a variety of species from bacteria to mammals. In the majority of cases, carbohydrate sulfation is implicated in regulating cellular interactions and cell adhesion (reviewed in 129). In eukaryotic cells, the most well studied example is that of adhesion mediated via the selectins and their sulfated ligands. P-selectin is expressed on activated endothelium and platelets, while L-selectin is expressed on circulating leukocytes. Selectins were named for the C-type lectin domains at their N-terminal regions which are responsible for binding to cell-surface carbohydrates, specifically, the tetrasaccharide sialyl LewisX. While the ligand for P-selectin, PSGL-1 is sulfated on tyrosine (130, 131), the L-selectin ligands, GlyCAM-1, CD34, and podocalyxin, are sulfated on O-linked oligosaccharides (132-134). However, for both P- and L-selectins, interactions with their ligands requires the sulfation of the cognate ligand (135-137). In addition, sulfation occurs constitutively on lymph node HEV to facilitate lymphocyte recirculation and it is tempting to speculate that sulfation could be induced at inflammatory sites to enable the initial interactions between leukocytes and endothelial cells.

The sulfation of proteins occurs in the Golgi apparatus, mainly in the last two of three cisternae on the trans side and in the trans Golgi network (138, 139). In the sulfation of
glycoconjugates or tyrosine residues on newly synthesized proteins, the high energy activated sulfate donor, adenosine 3’ phosphate 5’ phosphosulfate (PAPS), is used by all sulfotransferases to transfer a sulfate onto acceptor molecules. In the cytosol, ATP sulfurylase catalyzes the production of adenosine 5’ phosphosulfate (APS) from sulfate and ATP. APS is then used as a substrate for the APS kinase (ATP adenosine 5’ phosphosulfate 3’ phosphotransferase) which transfers a phosphate group from ATP to APS to yield PAPS which is then translocated to the lumen of the Golgi apparatus by a specific transporter (140). In mammals, PAPS synthesis is catalyzed by a family of bifunctional sulfurylase kinases (SK) that have both ATP-sulfurylase and adenosine-phosphosulfate kinase activities (reviewed in 141). The SK enzymes use a channeling mechanism to transfer the APS intermediate efficiently from the sulfurylase to the kinase active site. Mutation of one family member, SK2, results in the destruction of the channeling mechanism and the APS kinase active site in the brachymorphic mouse (142). Murine brachymorphism is characterized by severe skeletal defects attributed to undersulfation of cartilage proteoglycan. A nonsense mutation on an orthologous gene in humans results in spondyloepimetaphyseal dysplasia, a disorder affecting skeletal development (143).

Studies establishing the requirement for sulfation of selectin ligands for recognition by P- and L-selectin used chlorate, a selective inhibitor of the biosynthesis of PAPS (131, 136). Chlorate acts by inhibiting ATP sulfurylase, the first enzyme reaction in the synthesis of PAPS, thus removing a pool of sulfate donors for sulfotransferases to use. A number of sulfotransferases have been identified and they act on a variety of substrates with great specificity. While glycoconjugate sulfation is important for selectin ligand recognition, only recently have glycosyl sulfotransferases been cloned (144). Two of these enzymes were responsible for adding sulfate at the 6-position of Gal and GlcNac on O-linked oligosaccharides on L-selectin ligands expressed on HEV.

1.10 CD44 insolubility and associations with the cytoskeleton

In fibroblasts, a proportion of CD44 was insoluble after extraction in nonionic detergents,
particularly Triton X-100 (60, 85, 119, 145). This insolubility was interpreted to signify an association between CD44 and the cytoskeleton (reviewed in 146). However, it should be noted that CD44 from most epithelial cells and lymphocytes is entirely soluble in Triton X-100 (60, 147), indicating that association of CD44 with the detergent-insoluble cytoskeleton is cell-type specific. Enhanced HA binding by phalloidin-treated cells suggested that CD44 associated either directly or indirectly, with actin filaments (119). Agents responsible for actin depolymerization, such as cytochalasins and DNase I also affected CD44 solubility in Triton X-100, arguing for an association with the actin cytoskeleton (119, 128, 145). CD44 has also been shown to associate with ankyrin (58, 148-150), a cytoskeletal linker protein, and the ERM (ezrin, radixin, moesin) family of cytoskeletal proteins (151-153).

Some observations have been made however, that indicate that the insolubility of CD44 in nonionic detergents cannot be taken as evidence of an association with the cytoskeleton. In some cases, treatment of cells with cytoskeleton disrupting agents had no effect on the insolubility of CD44 in Triton X-100 (85, 154, 155). Perhaps the strongest data comes from cytoplasmic deletion mutants of CD44 transfected into fibroblasts which shows an identical extraction profile to the full length molecule in Triton X-100 (60, 155). Since most cytoskeletal interactions with CD44 are thought to occur via the CD44 cytoplasmic domain, these data suggest that cytoskeletal interactions may not be responsible for CD44 detergent insolubility.

1.11 Detergent insoluble low density lipid domains

The surfaces of most cells are studded with tiny, flask-shaped membrane invaginations which were first described in the 1950s as plasmalemmal vesicles. These compartments were originally identified as an endocytic compartment in endothelial cells that did not contain clathrin. One key characteristic of these compartments is that they are resistant to solubilization by nonionic detergents (reviewed in 156, 157, 158). Initially, detergent insolubility was used as a tool to investigate the sorting of proteins to the apical or basolateral side of epithelial cells and it was hypothesized that apical proteins sorted by virtue of an association with glycolipid “rafts” in the
Golgi complex that were impervious to detergent extraction. A simple procedure has been
developed to isolate these detergent resistant glycolipid rafts to facilitate characterization of proteins
that are enriched therein. Intact cells are treated with Triton X-100 on ice and the extract is
centrifuged to equilibrium on sucrose to separate the buoyant or low density fraction.

Low density lipid fractions isolated on sucrose gradients are enriched in certain lipids while
others are excluded. Low density fractions contain glycosphingolipids, such as \( \text{G}_{M1} \) and \( \text{G}_{M3} \) gangliosides, cerebrosides, sphingomyelin, and most importantly, cholesterol (159). By contrast,
very little glycerophospholipid (phosphatidyl ethanolamine, PE; phosphatidyl choline, PC; and
phosphatidyl inositol, PI) was found in the buoyant fractions of sucrose gradients. It is the
biophysical properties of these lipids that govern lipid domain formation, principally that
glycosphingolipids and sphingomyelin have a higher gel-to-liquid phase transition temperature than
glycerophospholipids. Glycosphingolipids are thought to exhibit stronger lateral cohesion as a
consequence of weak interactions between the sugar head groups and due to differences in length
and saturation of the acyl chains compared to glycerophospholipids (160). Since sphingolipid
head groups occupy a larger area than their associated acyl chains in the plane of the exoplasmic
leaflet of the plasma membrane, it is thought that cholesterol fills the void by acting as a spacer
between interacting glycosphingolipids (157). Thus, close-packed assemblies consisting of
sphingolipid and cholesterol cluster on the exoplasmic leaflet, while the remaining regions are filled
by unsaturated PC molecules. However, it must be noted that these “rafts” of lipids are dynamic,
with individual lipids moving in and out, which may explain why these glycosphingolipid-
cholesterol clusters are difficult to detect spectroscopically (161). It is only recently that the
existence of lipid rafts were demonstrated in living cells by chemical crosslinking and by the
measurement of energy transfer between fluorescently labeled membrane proteins (162, 163).

Detergent insoluble low density fractions contain abundant amounts of a 21 kD protein
called caveolin, thus giving rise to the term caveolae to refer to all low density domains (164, 165).
A subsequent study revealed that this protein is a member of a family of caveolins that have
restricted tissue specificities (166). Yet not all tissues and cells express caveolin, and detergent
insoluble lipid domains can be isolated from lymphocytes in the absence of caveolae (167). Furthermore, membrane fractions containing the bulk of detergent-insoluble GPI-linked protein can be separated from caveolin (168), indicating that “rafts” can exist inside and outside of caveolae (reviewed in 169). Many other proteins have been shown to associate with low density lipid rafts, including GPI-anchored proteins, various transmembrane proteins, src family kinases, G proteins, and some cytoskeletal proteins such as annexin II (170-173). For GPI-linked proteins, the lipid anchor determines association with low density lipid domains (174), while acylation of src family kinases, and the membrane spanning domain of transmembrane proteins (175) may determine partitioning with the buoyant density fraction.

The functions of sequestering certain proteins in detergent insoluble low density lipid fractions appear to be many and varied, probably reflecting the heterogeneity of these compartments. One proposed function of these fractions is in the biosynthetic pathway, by the formation of detergent insoluble complexes in the Golgi apparatus to direct the sorting of proteins destined for the apical surface in polarized cells (reviewed in 157, 158). Glycosphingolipid and cholesterol rich rafts are also implicated in the endocytic pathway which, in some cells, are equivalent to caveolae, whereas in others, no caveolae are present (176). In endothelial cells, caveolae are involved in transcytosis across the cell layer (177), and potocytosis (178), the transport small molecules into cells. Finally low density lipid rafts are also thought to be specialized signaling domains and have been identified in a number of different cells, including those that do not possess caveolae. GPI-anchored protein signal transduction is thought to occur in these compartments (179, 180). In mast cells, the FceRI signaling complex, including the src family kinase p53/56lyn, are sequestered in detergent-resistant membrane domains (181-184). Several molecules involved in TCR-mediated signaling are constitutively localized in the detergent resistant fractions of T cell lines, including the adaptor protein cbl, syk, LAT, and p21ras, while others proteins such as vav are recruited following stimulation through the TCR (185, 186), suggesting these signaling complexes are dynamic. Accessory molecules for TCR-mediated
signaling also localize to low density lipid fractions, including the GPI-linked CD48, (180), and CD28, which provides a co-stimulatory signal for T cell activation (187).

1.12 Summary of intentions

At the time that this work was initiated, the role of CD44 in leukocyte-endothelial cell interactions and the inflammatory response was not established and while numerous observations had been made regarding CD44 cytoskeletal interactions and the regulation of ligand binding, no unifying theories had emerged. CD44 insolubility had been reported by several groups, yet it had not been shown definitively that CD44 cytoskeletal interactions were the cause of its insolubility. Similarly, several factors had been shown to affect HA binding by CD44 but these effects often appeared to be specific to cell type, the developmental stage of the cell, or the activation state of the cell.

Several groups had reported that CD44 insolubility in Triton X-100 might be attributed to an association with the actin cytoskeleton. Yet, observations made by others indicated that CD44 did not associate with the cytoskeleton. One of the objectives of this thesis was to determine whether CD44 insolubility in Triton X-100 was caused by associations with low density lipids in NIH 3T3 cells. This was investigated using equilibrium density gradient centrifugation over sucrose to isolate the low density lipid fractions from cells. In addition to ascertaining whether CD44 could localize in these low density fractions, I also wanted to determine whether a particular region of the molecule was responsible for any observed localization to these fractions. A series of CD44 mutants and chimeric molecules transfected into NIH 3T3 cells were used to determine if a region of CD44 could mediate migration to low density lipid fractions.

Several factors had been identified that could regulate the ability of CD44 to bind its ligand HA, one being the post-translational modification of CD44. Both the modification of CD44 by N-linked and O-linked carbohydrate was shown to affect HA binding ability. The contribution of CD44 GAG modifications to HA binding was not very well defined especially in the context of CD44 glycosylation. In collaboration with Dr. F. Tufaro’s laboratory, a number of L cell variants
deficient in GAG synthesis were utilized to study the effect of GAGs on CD44-mediated HA binding. Furthermore, an L cell variant deficient in GAG synthesis and defective in glycosylation was used to examine the combined effects of these post-translational modifications on HA binding. Activation of cells either by alloantigen or PMA has been shown to induce CD44 to bind to HA in splenic T cells and T lymphoma lines. However, the biochemical events underlying this induction has not been well defined. Another aim of this work was to investigate whether pro-inflammatory soluble factors, such as TNFα, could induce HA binding by monocytes, a cell type involved in the propagation of the inflammatory response. Using the SR91 cell line, the ability of TNFα to induce HA binding and leukocyte-endothelial cell interactions was examined and the molecular mechanism of this induction was elucidated. Specifically, sulfation was investigated for its role in influencing the HA binding ability of SR91 cells and freshly isolated peripheral blood monocytes. In addition to TNFα, the effects of other pro-inflammatory factors on monocyte HA binding were evaluated to ascertain whether sulfation is a general mechanism by which CD44 adhesive function is induced. Ultimately, this information will help us to better understand how post-translational modification of the extracellular domain regulates CD44 adhesion and may give us some indications about the role of inducible sulfation at inflammatory sites.
CHAPTER 2

Materials and Methods

2.0 Cell Lines

The NIH 3T3 cells (188) were from the American Type Culture Collection (ATCC; Rockville, MD). The parental L cell line used was the clone 1D line of L M(tk') murine fibroblasts (ATCC, Rockville, MD). The mutant NIH 3T3 cell line transfected with the CD44.1 allele lacking a cytoplasmic tail was generated by Ruihong Li in the laboratory and L cells transfected with the chimeric CD44 molecule consisting of the CD44.1 allele containing the CD45 transmembrane domain (CD44-CD45 TM) was a generous gift of Dr. Jayne Lesley (55). The isolation of mutant L cell lines containing glycosaminoglycan deficiencies was described previously (189). Mutant cell lines used for experiments in chapter 4 were: gro2C (190), sog9 (191), and a ricin-resistant variant of sog9 (sog8) (192). SR91 cells, a human leukocytic cell line, were a generous gift of Dr. H. Klingemann (193). The murine endothelial cell line SVEC4-10 was a gift of Dr. G. Dougherty (194) and the myeloid progenitor cell line KG1a was obtained from the ATCC (Rockville, MD). The murine NIH 3T3 cells, SVEC4-10 cells, and L cell variants were maintained in Dulbecco’s modified eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) (Gibco BRL Life Technologies, Burlington, Ont.) and 50 U/ml of penicillin and streptomycin at 37°C, 5% CO₂. SR91 and KG1a cells were maintained in RPMI 1640 (Gibco BRL Life Technologies, Burlington, ON) supplemented with 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 5 X 10⁻⁵ M β-mercaptoethanol, and 50 U/ml of penicillin and streptomycin at 37°C, 5% CO₂.

2.1 Antibodies

Tissue culture supernatants (TCS) containing rat mAbs against murine CD44: IM7.8.1 (195), KM201 (196), or IRAWB 14 (15) were used for flow cytometry and immunoprecipitation, inhibition of HA binding, or flow cytometry and induction of HA binding, respectively and were
kindly given to me by Dr. J. Lesley and Dr. R. Hyman (IM7.8.1 and IRAWB 14) and Dr. P. Kincade (KM201). The rat mAb specific for the murine CD44.1 allele, RAMBM 44 was a gift of Drs. Hyman and Lesley (195). TCS containing mouse mAbs against human CD44, 3G12, 2G1, and 7F4 were a generous gift of Dr. G. Dougherty. The 3G12 mAb is able to block CD44-mediated FL-HA binding, 2G1 is specific for the CD44R1 isoform (197), and 7F4 binding to CD44 correlates with CD44-mediated HA binding (198). Rat mAbs used against murine Thy-1 and ICAM-1 were T24/37.1 (199) and YN1/1.7.4 (ATCC CRL 1878; 200) which were gifts from Dr. H.-S. Teh and Dr. F. Takei, respectively. The mouse anti-human CD34 mAb 8G12 was a gift from Dr. P. Lansdorp (201). Murine mAbs used against human CD43, H5H5 and the β1 integrin function blocking mAb, P5D2 (202) were obtained from the Developmental Studies Hybridoma Bank at the University of Iowa (DSHB; Iowa City, IA). Mouse anti-human VCAM-1 function blocking mAb was purchased from Serotec (Oxford, UK) and phycoerythrin (PE)-conjugated anti-human CD14 and anti-human ICAM-1 were purchased from Caltag Laboratories (Burlingame, CA). The polyclonal antibody against c-src, sc-18 was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), the anti-annexin II antisera was purchased from Transduction Laboratories (Lexington, KY) and the anti-actin mAb, clone C4 was from ICN Biomedicals Inc. (St. Laurent, PQ). Fluorescein isothiocyanate (FITC)-conjugated goat anti-rat and goat-anti-mouse antibodies were obtained from Jackson Immunoresearch Laboratories (Mississauga, ON) and horseradish peroxidase (HRP)-conjugated goat anti-rat antibodies were from Southern Biotechnology Associates, Inc. (Birmingham, AL). The antibodies and the concentrations at which they were used is listed in Table 2.1

2.2 Fluorescein conjugation of hyaluronan

Rooster comb hyaluronan (HA) was obtained as a sodium salt (Sigma-Aldrich Canada, Oakville, ON) and was conjugated to fluorescein according to the method of De Belder et al. (203). Briefly, 10 mg of rooster comb HA was dissolved in 8 ml of ddH₂O overnight at room temperature. The next day, 4 ml of dimethylsulfoxide (DMSO; Fisher Scientific, Nepean, ON)
### Table 2.1 Antibodies used for experiments

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Antigen</th>
<th>Flow Cytometry</th>
<th>Immunoprecipitation</th>
<th>Western blot</th>
<th>Binding assay</th>
<th>Source</th>
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<td>IM7.8.1</td>
<td>murine CD44</td>
<td>100 µl of TCS</td>
<td>100 µl TCS</td>
<td>1/20 TCS</td>
<td>3 µg/10⁶ cells</td>
<td>R. Hyman</td>
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<tr>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>A. Maiti</td>
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<td>ND</td>
<td>ND</td>
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<td>1/20 TCS</td>
<td>3 µg/well</td>
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<tr>
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<td>G. Dougherty</td>
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<td>1/500</td>
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<td>ND</td>
<td>1/5000</td>
<td>ND</td>
<td>Transduction</td>
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<tr>
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<td>murine actin</td>
<td>ND</td>
<td>ND</td>
<td>1/2000</td>
<td>ND</td>
<td>ICN</td>
</tr>
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</table>

*a amounts are expressed as a dilution factor of TCS or a concentration; *b as used in leukocyte-endothelial cell binding assays; *c amount used to immunoprecipitate from 5 x 10⁶ cell equivalents; *d ND, not done; *e blocking antibody incubated with SR91 cells; *f source/donor of antibody; *g dilution of TCS used in Western blots; *h blocking antibody incubated in each well of SVEC4-10 cells; *i Developmental Studies Hybridoma Bank at the University of Iowa; *j dilution of polyclonal antibody used in Western blots.
was added to the HA. The following were then combined in a glass vial: 300 µl of DMSO, 5 µl of acetaldehyde (Sigma-Aldrich Canada, Oakville, ON), 5 µl of cyclohexyl isocyanide (Sigma-Aldrich Canada, Oakville, ON), and 5 mg of fluoresceinamine isomer I (Sigma-Aldrich Canada, Oakville, ON), making sure the pH was between 5.0 and 7.0. The fluorescein mixture was then added to the HA and allowed to stir at room temperature for 5 hours in the dark. Fluoresceinated HA (FL-HA) was precipitated from free fluorescein by mixing with 160 ml of cold 100% ethanol and approximately 2 ml of saturated NaCl (~37% NaCl). The precipitate was isolated by centrifugation for 5 minutes at 900 g at 4°C. Precipitates were resuspended in 10 ml of ddH₂O, whereupon 100 ml of cold 100% ethanol with 2 ml of saturated NaCl were added and the solution centrifuged as above. This procedure was repeated until the supernatant was no longer orange due to the presence free fluoresceinamine. The pellet was dried under vacuum, weighed, and dissolved at a concentration of 1 mg/ml in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 12 mM Na₂HPO₄, 1.8 mM KH₂PO₄). The FL-HA was titrated on cells at concentrations of 1 µg/ml to 200 µg/ml by flow cytometry and the optimal concentration was determined to be 15 µg/ml for one batch and 5 µg/ml for another. Concentrations of FL-HA used in each experiment are indicated in the figure legends or in the appropriate section of this chapter.

2.3 Purification of the anti-CD44 mAb IM7.8.1

The IM7.8.1 hybridoma cell line was grown in 500 ml of DMEM/10% FCS in roller bottles at 37°C, 5% CO₂ for 2 weeks. The TCS was separated from the cells by centrifugation, filtered over 0.2 micron cellulose acetate and the pH adjusted to 8.0 by the addition of 1/10 volume of 1 M Tris pH8.0. All subsequent steps took place at 4°C. The TCS was loaded by gravity onto a 5 ml packed Protein G Sepharose 4-Fast Flow column, the column was washed with 15 column volumes of borate buffer (0.1 M borate, 0.5 M NaCl, 2.5 mM EDTA) until the A_280 of the flow through was close to zero. The mAb was eluted with 15 ml of 0.1 M glycine pH2.5 and 1 ml fractions were collected and neutralized with saturated Tris. Based on their A_280 fractions containing protein were pooled, dialyzed for 2 days against 2 changes of 1.5 litres of PBS, and the
mAb concentration was determined by the calculation that an $A_{280}$ of 1.46=1 mg/ml of purified antibody.

2.4 Coupling IM7.8.1 to activated CNBr-Sepharose

An appropriate amount of freeze-dried activated CNBr-Sepharose (Amersham Pharmacia Biotech Ltd., Baie d’Urfe, PQ) was rehydrated in a sintered glass funnel lined with a Metricel filter (Gelman Sciences Inc., Ann Arbor, MI) over vacuum with 200 ml/g of 1 mM HCl and then placed in a 50 ml Falcon tube with coupling buffer (0.1 M NaHCO$_3$, pH8.3, 0.5 M NaCl). Resuspension of 1 g of CNBr-Sepharose resulted in approximately 3.5 ml of swollen gel and 4 mg of antibody were coupled to 1 ml of swollen gel. Approximately 20 mg of purified IM7.8.1 mAb was dialyzed against coupling buffer, added to the CNBr-Sepharose and allowed to rotate end-over-end at room temperature for 2 hours. The tube was centrifuged at 1000 g at 4°C and then transferred to the sintered glass funnel lined with a new Metricel filter. The CNBr-Sepharose mixture was washed with 3 cycles of alternating pH, starting with acetate buffer (0.1 M sodium acetate pH4.0, 0.5 M NaCl) and followed by Tris buffer (0.1 M Tris pH8.0, 0.5 M NaCl). The CNBr-IM7.8.1 was resuspended in phosphate buffered saline (PBS) as a 50% slurry, and titrated for its ability to immunoprecipitate CD44.

2.5 Biotinylation of IM7.8.1

Approximately 2 mg of purified IM7.8.1 was dialyzed against 0.1 M NaHCO$_3$, pH8.0 and the concentration was adjusted to 1 mg/ml by taking an $A_{280}$ reading. Biotin (Biotinamidicaproate N-hydroxysuccinimide ester; Pierce, Rockford IL) was dissolved in DMSO at a concentration of 1 mg/ml and 120 µl was added per mg of purified antibody and incubated at room temperature, rotating end-over-end for 4 hours. The biotinylated mAb was then dialyzed against 3 changes of 1.5 litres of PBS. $A_{280}$ was used to determine the final concentration of the mAb where an $A_{280}$ of 1.46=1 mg/ml. The biotinylated IM7.8.1 was titrated against CD44-positive cells by flow
cytometry with FITC-conjugated streptavidin or on western blots against CD44-positive cell lysates with HRP-conjugated streptavidin.

2.6 Triton X-100 lysis of NIH 3T3 cells

Monolayers of NIH 3T3 and L cells were removed from dishes with 1 mM EDTA in PBS at 37°C for 5 minutes. Cells were washed in PBS and lysed with 25 μl of ice cold 1% Triton X-100 (Fisher Scientific, Nepean, ON) TNE lysis buffer (1% Triton X-100, 10 mM Tris pH7.5, 150 mM NaCl, 2 mM EDTA) containing 200 μM phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich Canada, Oakville, ON), 1 μg/ml of leupeptin (Roche Diagnostics, Laval, PQ), 1 μg/ml of aprotinin (Roche Diagnostics, Laval, PQ), and 1 μg/ml of pepstatin (Roche Diagnostics, Laval, PQ) on ice for 10 minutes. Lysates were clarified by centrifugation at 12 000 g at 4°C for 10 minutes, supernatants were resuspended in an equal volume of 2X non-reducing sample buffer, boiled, and separated on 7.5% polyacrylamide gels.

2.7 Detection of proteins by western blot

In general, proteins separated by SDS-PAGE were transferred to polyvinylidene difluoride (PVDF) Immobilon-P membranes (Millipore Canada Ltd., Mississauga, ON) in a Biorad Mini Protean II system (Biorad Laboratories Canada Ltd., Mississauga, ON) at 100 V for 1 hour. The membrane was allowed to dry at room temperature for 1 hour prior to detection with antibodies. Primary antibodies were diluted at concentrations indicated in Table 2.1 or the figure legends in 5% skim milk powder, 20 mM Tris pH7.5, 150 mM NaCl, 0.1% Tween-20 (TBST) and the membrane incubated for 1 hour, washed with 2 changes of TBST, and incubated with a 1/5000 dilution of HRP-conjugated goat anti-rat Ig, protein A, or goat-anti-mouse Ig in 5% skim milk-TBST for 30 minutes. Membranes were washed thoroughly with several changes of TBST and developed using the enhanced chemiluminescence assay according to the manufacturer's instructions (ECL kit; Amersham Pharmacia Biotech, Baie d'Urfé, PQ). In all cases, prestained
molecular weight markers (New England Biolabs, Mississauga, ON.) were run on the SDS polyacrylamide gels and transferred to the PVDF membrane.

2.8 Immunofluorescence microscopy of NIH 3T3 cells

Approximately $1 \times 10^4$ NIH 3T3 cells in 100 $\mu$l of DMEM/10% FCS were seeded onto 22 mm$^2$ square coverslips in a well of a 6-well dish and allowed to adhere for 3 hours after which the well was filled with 2 ml of DMEM/10% FCS and the cells incubated overnight. To label, cells were washed twice with 2 ml of PBS and either incubated in 100 $\mu$l of PBS/2% FCS alone or in 10 $\mu$l of 0.5% Triton X-100 in PBS for 30 minutes on ice. All subsequent steps were carried out on ice. The coverslips were washed once in 2 ml of PBS and fixed with 100 $\mu$l of 4% paraformaldehyde in PBS for 30 minutes, then blocked with 100 $\mu$l of 10 mM glycine in PBS/2% FCS for 20 minutes and washed twice with 2 ml of PBS/2% FCS. Cells were blocked by incubation in 100 $\mu$l of PBS/2% FCS for 30 minutes. To label CD44, 100 $\mu$l of the mAb IRAWB 14 TCS was added to the coverslip for 30 minutes. The cells were washed twice with 2 ml of PBS/2% FCS and incubated with 100 $\mu$l of FITC-conjugated goat-anti-rat Ig diluted 1/50 in PBS/2% FCS for 30 minutes on ice. Coverslips were washed twice with 2 ml of PBS/2% FCS, once with 2 ml of PBS, excess PBS was blotted from the cell free side of the coverslip and mounted on a slide with a drop of Fluoromount (Gibco BRL Life Technologies, Burlington, ON) containing 2% 2, 4 diazo bicyclo [2-2-2] octane (DABCO; Sigma-Aldrich Canada, Oakville, ON). Cells were visualized on a Zeiss axiophot immunofluorescent microscope (Carl Zeiss Canada Ltd., Don Mills, ON) under an oil immersion lens at 1000X magnification.

2.9 Isolation of the low density lipid fraction by density gradient centrifugation on sucrose

Total Triton X-100 or n-octyl-β-D-glucopyranoside (Calbiochem, La Jolla, CA) lysates of NIH 3T3 cells, L cells, or transfected NIH 3T3 or L cells were separated on sucrose density gradients according to the method of Rodgers et al. (204). Cells from two confluent 100 mm$^2$
plates (approximately 5 X 10⁶ cells) were rinsed in PBS and the plates placed on ice for 10 minutes in a total of 1 ml of 1% Triton X-100-TNE or 60 mM n-octyl-β-D-glucopyranoside-TNE lysis buffer containing protease inhibitors (see section 2.6). The cells were scraped off the plates with a cell scraper on ice and the resulting lysate was homogenized with 10 strokes of a Dounce homogenizer on ice. The total lysate was adjusted to 40% sucrose by dilution in an equal volume of 80% (w/v) sucrose-TNE (80% sucrose w/v, 10 mM Tris pH7.5, 150 mM NaCl, 2 mM EDTA) containing protease inhibitors. The diluted lysate was placed at the bottom of a Beckman SW41 Ultraclear tube (cat# 344059; Beckman Instruments Inc., Mississauga, ON) and 6 ml of 30% (w/v) sucrose-TNE followed by 3.5 ml of 5% (w/v) sucrose-TNE were layered above it with a 10 ml syringe fitted with an 18 gauge needle. The samples were centrifuged in a Beckman SW41 rotor at 39,000 rpm (>250,000 g) for 14 hours at 4°C. Following centrifugation, 1.5 ml fractions were collected from the top of the gradient. Fractions 5-8, which generally contain over 20% sucrose (204) were diluted with an equal volume of ddH₂O. Proteins were precipitated on ice in a final concentration of 10% TCA for 30 minutes, centrifuged and washed twice in 100 μl of ice cold acetone. Protein pellets were air dried for 2 minutes after the final acetone wash and either resuspended in sample buffer for SDS-PAGE or resuspended in 1 ml of 1% Triton X-100-TNE in preparation for immunoprecipitation of CD44.

2.10 Flow cytometry of adherent cell lines

Approximately 1-2 X10⁶ adherent cells were removed from dishes with 1 mM EDTA in PBS and labeled with 100 μl of TCS or 2 μg/ml of purified mAbs for 20 minutes on ice. Cells were washed with PBS containing 2% FCS and 2 mM EDTA pH8.0. Cells were then incubated in a 1/100 dilution of FITC-conjugated goat anti-rat or goat-anti-mouse antibodies for 20 minutes on ice, washed, and resuspended in PBS/2% FCS/2 mM EDTA containing 5 μg/ml of propidium iodide (Sigma-Aldrich Canada, Oakville, ON). Adherent cells were incubated for 5 minutes with 100 μl of IRAWB 14 or KM201 TCS or with buffer alone to either induce FL-HA binding, prevent FL-HA binding, or as a control, respectively. The cells were then incubated with 15 μg/ml
of FL-HA for 20 minutes on ice and washed. To block IRAWB 14 induced FL-HA binding, cells were first pretreated for 5 minutes with 100 µl of KM201 TCS, then washed prior to treatment with 100 µl of IRAWB 14 TCS for 5 minutes followed by incubation for 20 minutes with FL-HA. All labeled cells were analyzed on a FACScan® flow cytometer (Becton Dickinson, Mississauga, ON) using Lysis II® software. For inhibition of sulfo-transferase activity, cells were incubated with 10 mM sodium chlorate (Sigma-Aldrich Canada, Oakville, ON) for 2 days in DMEM supplemented with 10% FCS at 37°C, 5% CO₂.

2.11 Detection of CD44 from L cell variants by western blot

5 X 10⁵ cells were removed from plates by incubation in 1 mM EDTA in PBS at 37°C for 5 minutes after which cells were pipetted into tubes. Cells were washed once in PBS and lysed in 60 mM n-octyl-β-D-glucopyranoside (Calbiochem, La Jolla, CA), 10 mM Tris pH7.5, 150 mM NaCl, 2 mM EDTA, 200 µM PMSF, 1 µg/ml of leupeptin, 1 µg/ml of aprotinin, and 1µg/ml of pepstatin (lysis buffer) and incubated on ice for 10 minutes. Lysates were clarified by centrifugation at 12 000 g at 4°C for 10 minutes, supernatants were resuspended in an equal volume of 2X non-reducing sample buffer, boiled, separated on 7.5% polyacrylamide gels and transferred to an Immobilon-P membrane at 100 V for 1 hour. Dried membranes were incubated with a 1/20 dilution of KM201 TCS in 5% skim milk powder, 20 mM Tris pH7.5, 150 mM NaCl, 0.1% Tween-20 (TBST) for 1 hour, washed, and then incubated with a 1/5000 dilution of HRP-conjugated goat anti-rat Ig in 5% skim milk-TBST for 30 minutes. Membranes were washed thoroughly and developed using the ECL assay according to the manufacturer's instructions (Amersham Pharmacia Biotech, Baie d'Urfé, PQ). In all cases, prestained molecular weight markers (New England Biolabs, Mississauga, ON) were run on the SDS polyacrylamide gels and transferred to the PVDF membrane.
2.12 Sulfate labeling and treatment of L cells with chondroitin ABC lyase

Three sub-confluent 100mm² plates of L cells (5 X 10⁶ cells/plate) were each incubated with 200 µCi of [³⁵S]-sulfate (specific activity ~43 Ci/mg, ICN Biomedicals Inc., St. Laurent, PQ) in 4 ml of DMEM/2% FCS for 3 days. Cells were washed with 5 ml of PBS and incubated with 1 ml of 60 mM n-octyl-β-D-glucopyranoside-TNE on ice for 5 minutes and then scraped off the plates with a cell scraper. Lysates were pooled, centrifuged at 12,000 g for 10 minutes, and then precleared three times with 30 µl of a 50% slurry of Sepharose CL-4B (Sigma-Aldrich Canada, Oakville, ON) for 1 hour at 4°C, rotating end over end. IM7.8.1 mAb (100 µl TCS) was precoupled to 20 µl of Protein G Sepharose-4 Fast Flow (Amersham Pharmacia Biotech, Baie d’Urfé, PQ) for 2 hours, washed, and added to the precleared lysates for 2 hours at 4°C, rotating end over end. Immunoprecipitates were washed three times in lysis buffer and divided into two samples containing 25 µl of 0.01% BSA, 40 mM Tris, 40 mM sodium acetate, pH8.0, one of which was treated with 15 µl (15 mU) of chondroitin ABC lyase (Roche Diagnostics, Laval, PQ). Samples were incubated for 1 hour at 37°C before 3X reducing sample buffer was added, the samples boiled and loaded on a 7.5% reducing SDS-PAGE gel. Gels were treated with Amplify® (Amersham Pharmacia Biotech, Baie d’Urfé, PQ), dried, and exposed at -70°C with Kodak BioMax MR film (InterScience, Markham, ON) with an intensifying screen for 14 days.

2.13 Pulse-chase of L cells and sog8 cells and treatment with endoglycosidase H

One confluent 60 mm² plate (~3 X 10⁶ cells) of L cells and sog8 cells per time point was washed with PBS and then starved of methionine and cysteine by incubation in 2 ml of DMEM methionine(-) and cysteine(-) media (ICN Biomedicals Inc., St. Laurent, PQ) for 45 minutes at 37°C. The cells were then labeled for 20 minutes at 37°C with 250 µCi of [³⁵S]-Express (specific activity >3000 Ci/mmol, 70% methionine, 30% cysteine; NEN DuPont Canada, Markham, ON) in 2 ml of methionine and cysteine deficient DMEM supplemented with 1% Glutamax I (Gibco BRL Life Technologies, Burlington, ON). Cells were either harvested immediately or washed in 5 ml
PBS and incubated in DMEM/10% FCS for 15, 30, 60 or 120 minutes. Cells were lysed as in section 2.12. A small aliquot of each lysate was precipitated in 10% TCA to determine the amount of radiolabel incorporated into each cell line and lysates containing equivalent amounts of radioactivity were used for immunoprecipitation. Lysates were immunoprecipitated as above (section 2.12) then washed twice in high salt buffer (1% Nonidet P-40, 10 mM Tris pH7.5, 500 mM NaCl, 2 mM EDTA) three times in low salt buffer (1% Nonidet P-40, 10 mM Tris pH7.5, 150 mM NaCl, 2 mM EDTA), and once in 10 mM Tris pH7.5 and divided in two. Half of the immunoprecipitate was digested by the addition of 12 µl of 85 mM sodium citrate pH 5.5, 2 µl (2 mU) of Endoglycosidase H (endoH; Boehringer Mannheim Canada, Laval, PQ) and 2 µl of 0.1 M PMSF and incubated at 37°C overnight. The other half of the immunoprecipitated material was incubated at 37°C overnight in 85 mM sodium citrate pH 5.5 and 2 µl of 0.1 M PMSF without endoH. All immunoprecipitates were boiled in an equal volume of 2X reducing sample buffer and separated on 7.5% reducing SDS-PAGE gels. Samples were treated as in section 2.12 except gels were exposed for 2 days.

2.14 Stimulations of SR91 cells and flow cytometry

SR91 cells (10⁶ cells/ml) were stimulated with TNFα (10 ng/ml), IFNγ (500 U/ml), LPS (1 µg/ml), IL-1β (10 ng/ml), MIP-1α (50 ng/ml), or MCP-1 (50 ng/ml) in RPMI 1640/10% FCS for 24 hours at 37°C, 5% CO₂. All cytokines and chemokines were purchased from R & D Systems (Minneapolis, MN) with the exception of LPS which was a gift from Dr. H.-S. Teh (Sigma-Aldrich Canada, Oakville, ON). Sodium chlorate (Sigma-Aldrich Canada, Oakville, ON) made in RPMI 1640 was added concurrent with the cytokine treatment at concentrations indicated in the figure legends. The protocol for labeling of cells for flow cytometry was as described in section 2.10. Briefly, 100 µl of TCS or 2 µg/ml of purified mAbs were used to label surface molecules. Depending on the primary mAb used, a 1/100 dilution of FITC-conjugated goat-anti-rat Ig or goat-anti mouse Ig was used. FL-HA was used at a concentration of 5 µg/ml. To block FL-HA binding, SR91 cells were incubated with 100 µl of IM7.8.1 TCS for 15 minutes or 50
μg/ml of freshly made rooster comb HA in PBS, washed in PBS/2% FCS/2 mM EDTA, and incubated with 5 μg/ml of FL-HA.

2.15 Sulfate labeling of SR91 cells and CD44 immunoprecipitations

SR91 cells at a concentration 10⁶ cells/ml were labeled with 100 μCi/ml of [³⁵S]sulfate (~43 Ci/mg; ICN Biomedicals Inc., St. Laurent, PQ) in RPMI 1640 medium containing 2% FCS for 24 hours at 37°C, 5% CO₂. For each sample, 5 X 10⁶ cells were treated with cytokine or chlorate, as described in section 2.14. Cells were then washed twice with PBS and lysed in 1 ml of 1% Triton X-100-TNE containing protease inhibitors (as in section 2.6), precleared with 30 μl of a 50% slurry of Sepharose CL-4B (Sigma-Aldrich Canada, Oakville, ON). CD44 was immunoprecipitated using CNBr-coupled IM7.8.1 and separated on a 7.5% reducing or non-reducing SDS-PAGE gels, as described in figure legends. Gels were either dried and exposed to Kodak BioMax MR film for autoradiography or transferred to a membrane and immunoblotted with 2 μg/ml of biotinylated IM7.8.1 followed by a 1/5000 dilution of HRP-conjugated streptavidin. Membranes were developed by ECL for visualization on Kodak BioMax MR film.

2.16 Leukocyte-endothelial cell binding assays

Unstimulated and stimulated SR91 cells (10⁶ cells/ml) were resuspended in CellTracker® Green 5-chloromethyl fluorescein diacetate (CMFDA; Molecular Probes, Portland, OR) in RPMI 1640 for 30 minutes at 37°C. Cells were resuspended in RPMI 1640/10% FCS for 30 minutes at 37°C, and washed twice with PBS/2% FCS. SR91 cells (3 X 10⁵) were incubated with a confluent monolayer of murine SVEC4-10 cells in 0.5 ml of PBS/2% FCS for 30 minutes at room temperature and washed four times with PBS. Cells were fixed in 4% paraformaldehyde in PBS and analyzed on a Cytofluor 2300 fluorimeter (Millipore Canada Ltd., Mississauga, ON) or photographed (Zeiss Axiophot microscope; Carl Zeiss Canada Ltd., Don Mills, ON). For inhibition studies, cells were pretreated with 3 μg of purified anti-CD44 mAbs IM7.8.1 and KM201 or 1 μg of anti-ICAM-1 in PBS/2% FCS, 50 μg/ml of rooster comb HA (Sigma-Aldrich...
Canada, Oakville, ON), or 5 μg/ml of ovine hyaluronidase (Calbiochem, La Jolla, CA) and incubated for 20 minutes at 4°C after the CMFDA labeling period. CMFDA-labeled SR91 cells (3 X 10⁵; 100% input cells) and wells containing the monolayer alone were used to determine 100% binding and background binding levels respectively.

2.17 Digests of CD44 immunoprecipitates to remove glycosaminoglycans and oligosaccharides

[^35]S sulfate labeled CD44 immunoprecipitated from 5 X 10⁶ unstimulated and stimulated SR91 cells with CNBr-coupled IM7.8.1 was incubated in 100 μl of 1% SDS, 10 mM Tris pH7.5 at 100°C to denature CD44, aliquoted into tubes, and precipitated with 8 volumes of ice cold acetone for 2 hours to overnight at -20°C. Acetone precipitated CD44 was centrifuged, washed twice in 100 μl of acetone, air dried for 2 minutes and resuspended in the appropriate buffers for subsequent enzymatic digestions. For[^35]S methionine and cysteine labeling, SR91 cells at a density of 10⁶/ml were labeled with 100 μCi/ml of[^35]S-Express (specific activity >3000 Ci/mmol, 70% methionine, 30% cysteine; NEN DuPont Canada, Markham, ON) in DMEM methionine(-) cysteine(-) media containing 2% FCS and 1% Glutamax overnight at 37°C, 5% CO₂. For biotinylation, cells were washed in PBS/1% glucose (w/v) 3 times after[^35]S sulfate labeling, resuspended in 1 ml of PBS/1% glucose (w/v) and biotinylated with 1mg of a 1 mg/ml solution of ImmunoPure® Sulfo-NHS-Biotin (Sulfosuccinimidobiotin; Pierce, Rockford, IL) for 30 minutes on ice, mixing occasionally. Cell were washed 4 times with PBS containing 1% glucose (w/v), 1 mg/ml of BSA, and 2 mg/ml of lysine prior to lysis. CD44 from SR91 cells was subjected to the following digestions to remove glycosaminoglycans and carbohydrate moieties. To remove heparan sulfate, acetone precipitated CD44 was resuspended in 40 μl of heparitinase buffer (17 mM Tris pH7.5, 42 mM NaCl, 3 mM CaCl₂, 0.01% BSA), containing 2 μl of 0.1 M PMSF and 4.5 μl (1.125 U) of heparitinase II (Seikagaku America Inc., Ijamsville, MD) and incubated at 37°C for 1 hour. Keratan sulfate was removed from CD44 by incubating immunoprecipitates in 20 mM Tris pH7.2, 2 μl of 0.1 M PMSF, and 1 μl (50 mU) of keratanase (Seikagaku America Inc.,
Ijamsville, MD), alone or in combination with 4 µl (2 mU) keratanase II (Seikagaku America Inc., Ijamsville, MD) at 37°C, overnight. Hyaluronidase digestions were carried out in 100 µl of hyaluronidase buffer (20 mM sodium acetate pH6.0, 150 mM NaCl) containing 2 µl of 0.1 M PMSF and 4 µl (2 U) of hyaluronidase from *Streptomyces hyalurolyticus* (Calbiochem, La Jolla, CA) at 60°C for 30 minutes. Chondroitin sulfate was digested from CD44 immunoprecipitates as in section 2.12. N-linked oligosaccharides were digested by incubation in the G7 buffer provided by the manufacturer, containing 1% NP-40, 2 µl of 0.1 M PMSF and 4 µl (2000 U) peptide N-glycosidase F (PNGaseF; New England Biolabs, Mississauga, ON) at 37°C, overnight. O-linked oligosaccharides were removed from CD44 by digestion with neuraminidase to remove sialic acid prior to O-glycanase digestion. Briefly, acetone precipitated CD44 was resuspended in 40 µl of 50 mM sodium acetate, 4 mM CaCl$_2$ pH7.8 containing 2 µl of 0.1 M PMSF and 4 µl (4 mU) of neuraminidase from *Vibrio cholerae* (Roche Diagnostics, Laval, PQ) and incubated at 37°C for 4 hours. The neuraminidase digested material was precipitated with 8 volumes of ice cold acetone, washed with acetone, dried and resuspended in 100 µl of 20 mM sodium cacodylate, 20 mM NaH$_2$PO$_4$ pH6.5 containing 2 µl of 0.1 M PMSF and 4 µl (2 mU) of BSA-free O-glycosidase (Roche Diagnostics, Laval, PQ) and incubated at 37°C, overnight. All digests were precipitated in 8 volumes of ice cold acetone, washed twice in acetone, air dried, resuspended in reducing sample buffer, separated on 7.5% reducing SDS-PAGE gels, transferred to Immobilon-P membranes (Millipore Canada Ltd., Mississauga, ON) and exposed to a phosphoimager cassette for 3 days and analyzed using ImageQuant software (Molecular Devices, Sunnyvale, CA) or exposed to Kodak BioMax MR film (Interscience, Markham, ON) at -70°C with an intensifying screen for 5-14 days for autoradiography.

### 2.18 Removal of O-linked oligosaccharides by β-elimination

[$^{35}$S]sulfate labeled CD44 immunoprecipitated from 5 X 10$^6$ TNFα stimulated SR91 cells with CNBr-coupled IM7.8.1 was incubated in 100 µl of 1% SDS, 10 mM Tris pH7.5, at 100°C to denature CD44 and precipitated with 8 volumes of ice cold acetone at -20°C for 2 hours to
overnight. Precipitated CD44 was resuspended in 0.1 M NaOH, 1 M NaBH₄ (Fisher Scientific, Nepean, ON) and incubated at 45°C for 24 hours in a β-elimination reaction. The reaction was neutralized with a few drops of glacial acetic acid and the pH was confirmed to be between 6.5 and 7. The neutralized solution was then precipitated in 8 volumes of ice cold acetone at -20°C for 2 hours, washed twice in acetone, air dried, and resuspended in reducing sample buffer, separated on 7.5% reducing SDS-PAGE gels, transferred to Immobilon-P PVDF membranes (Millipore Canada Ltd., Mississauga, ON) and exposed to a phosphoimager cassette for 3 days and analyzed using ImageQuant software (Molecular Devices, Sunnyvale, CA) or exposed to Kodak BioMax MR film (Interscience, Markham, ON) at -70°C with an intensifying screen for 5-14 days for autoradiography.

2.19 Sulfo-tyrosine analysis of CD44 by aryl sulfatase digestion, 1D, and 2D thin layer electrophoresis

To enzymatically remove sulfate from tyrosines, [³⁵S]sulfate labeled CD44 immunoprecipitated from 5 X 10⁶ unstimulated and stimulated SR91 cells with protein G sepharose-coupled IM7.8.1 was resuspended in 40 μl of 10 mM Tris pH7.5, 2 μl of 0.1 M PMSF, and 30 μl (500 mU) of aryl sulfatase from Aerobacter aerogenes (Sigma-Aldrich Canada, Oakville, ON) and incubated at 37°C, overnight. An equal volume of 2X reducing sample buffer was added to the sample which was then boiled and separated on 7.5% reducing SDS-PAGE gels. Gels were fixed in 40% methanol, 10% acetic acid, treated with Amplify® (Amersham Pharmacia Biotech, Baie d’Urfé, PQ), dried and exposed at -70°C onto Kodak Biomax MR film (Interscience, Markham, ON) with an intensifying screen for 10 days.

One dimensional thin layer electrophoresis (TLE) was carried out in the following way. [³⁵S]sulfate labeled CD44 immunoprecipitated from 5 X 10⁷ TNFα stimulated SR91 cells with protein G sepharose-coupled IM7.8.1 was resuspended with an equal volume of 2X reducing sample buffer, separated on a 7.5% reducing SDS-PAGE gel, transferred to an Immobilon-P membrane and exposed to a phosphoimager cassette for 3 days. Based on analysis on ImageQuant.
software (Molecular Dynamics, Sunnyvale, CA) the $[^{35}\text{S}]$sulfate labeled CD44 band was excised, washed once in methanol, twice in ddH$_2$O, chopped into small pieces and treated with barium hydroxide or pronase. Excised membranes were placed in a 1 ml reactivial (Pierce, Rockford, IL) with 1 ml of 0.2 M Ba(OH)$_2$, a stream of nitrogen was blown over the vial prior to sealing, and the protein hydrolyzed at 110°C for 24 hours. After Ba(OH)$_2$ hydrolysis, samples were placed on ice, the seal broken and the sample transferred to eppendorf tubes. The pellet was extracted with 400 µl of ddH$_2$O and neutralized with 5 drops of 1 M H$_2$SO$_4$ and a few drops of 0.1 M H$_2$SO$_4$ until the solution had reached pH 7.0. To treat membranes with pronase, 150 µg of pronase (Roche Diagnostics, Laval, PQ) was prepared in 50 mM NH$_4$HCO$_3$ with a trace of phenol red at a concentration of 50 µg/ml and preincubated for 1 hour at 37°C prior to addition to the membranes. Pronase digestion of the excised membrane followed at 37°C, overnight. The eluates from the Ba(OH)$_2$ hydrolysis and from the pronase digestion were lyophilized, resuspended in 30 µl of the TLE buffer (7.8% acetic acid, 2.2% formic acid, trace phenol red), spotted onto a silica gel TLE plate (VWR Canlabs, Mississauga, ON), dried with a hair dryer, and run at 750 V for 110 minutes. Sulfo-tyrosine (Senn Chemicals AG, Dielsdorf, Switzerland) and phospho-tyrosine (Sigma-Aldrich Canada, Oakville, ON) standards were made in the first dimension TLE buffer with phenol red at a concentration of 1 µg/ml and 6 µg of standard was spotted onto the plate and run concurrently with the samples. After electrophoresis, the plate was dried with a hair dryer, baked at 40°C for 10 minutes and sprayed with 0.2% ninhydrin to mark the standards. To develop radioactive spots, the TLE plate was placed on a phosphoimager cassette for one week and developed using ImageQuant software or placed on Kodak BioMax MR film for one to two months at -70°C for autoradiography.

To analyze CD44 by 2D-TLE, CD44 was immunoprecipitated from 3 X $10^9$ unstimulated and TNFα stimulated SR91 cells labeled with $[^{35}\text{S}]$sulfate. The immunoprecipitate was resuspended in 2X reducing sample buffer, separated on a 7.5% reducing SDS-PAGE gel, transferred to a PVDF membrane and exposed to Kodak BioMax MR film. Based on autoradiography, the $[^{35}\text{S}]$sulfate labeled CD44 bands were excised from the membrane, washed
once in methanol, twice with ddH$_2$O, chopped up, and placed in a reactivial. Alkaline hydrolysis of the membrane pieces ensued as described above with 500 µl of 0.2 M Ba(OH)$_2$ for 24 hours at 110°C. After hydrolysis, the samples were neutralized and applied to the TLE plate (VWR Canlab, Mississauga, ON) as described above. After electrophoresis in the first dimension at 750 V for 110 minutes, the plate was dried with a hair dryer, and rewet with the second dimension buffer (5% acetic acid, 0.5% pyridine), turned 90° clockwise and run at 1000 V for 50 minutes. Preparation of sulfo-tyrosine and phospho-tyrosine standards and development of the plate after electrophoresis was as described above for the 1D-TLE analysis.

2.20 Isolation of peripheral blood mononuclear cells from human blood

To isolate human peripheral blood mononuclear cells (PBMCs), approximately 40 ml of blood was collected from donors in 4 heparin Vacutainer tubes (Becton-Dickinson, Mississauga, ON) by phlebotomists at Vancouver General Hospital, UBC site. Heparinized blood was diluted with 3 volumes of Hanks' balanced salt solution (HBSS; Gibco BRL Life Technologies, Burlington, ON). Ficoll-Paque® Plus (Amersham Pharmacia Biotech, Baie d'Urfé, PQ) was brought to room temperature and 10 ml was layered at the bottom of a 50 ml Falcon tube. The diluted heparinized blood (~40 ml) was slowly layered over each of the Ficoll-hypaque containing tubes at a 45° angle and centrifuged at 400 g for 40 minutes at room temperature without the brake. The upper layer containing plasma and platelets was removed with a 10 ml pipet and using a 5 ml pipet, the buffy coat containing PBMCs was removed to a new tube. This was repeated for all the Ficoll-hypaque tubes and the buffy coats were pooled. The PBMCs were washed 3 times with 25 ml of HBSS by centrifugation at 900 g for 10 minutes at room temperature. The PBMC pellet was resuspended in RPMI/10% FCS and counted on a haemocytometer in preparation for stimulations with various factors.

2.21 Isolation of monocytes and macrophages from PBMCs

To isolate monocytes and macrophages from donor blood, PBMCs were isolated on Ficoll-
Paque® Plus as described above, resuspended in RPMI 1640 containing 10% FCS and counted. Approximately 1 X 10^7 PBMCs at a density of 10^7 cells/ml were plated in each well of a Nunc 6-well plate (Gibco BRL Life Technologies, Burlington, ON) and the monocyte/macrophage subpopulation was allowed to adhere at 37°C, 5% CO₂, for 45 minutes. The non-adherent lymphocytes were aspirated and the adherent cells were washed twice with PBS. Fresh RPMI/10% FCS was added to the adherent cells which were allowed to rest for 1 hour at 37°C, 5% CO₂ prior to stimulations. Stimulations were carried out in 5 ml of RPMI/10% FCS.

2.2.2 Stimulation of PBMCs and flow cytometry

Approximately 5 X 10^6 PMBCs or monocytes and macrophages purified by adherence to plastic (10^6 cells/ml) were stimulated with TNFα (10 ng/ml), IL-1β (10 ng/ml), MIP-1α (50 ng/ml), or MCP-1 (50 ng/ml) in 5 ml of RPMI 1640/10% FCS for 72 hours at 37°C, 5% CO₂. All cytokines and chemokines were purchased from R & D Systems (Minneapolis, MN). Sodium chlorate (50 mM) made in RPMI 1640 was added concurrent with the cytokine treatment at concentrations indicated in the figure legends. Labeling of cells for flow cytometry was as described in section 2.10. Briefly, cells were incubated in 100 µl of a 1/10 dilution of normal mouse serum (Cedarlane Ltd., Hornby, ON) in PBS/2% FCS for 15 minutes on ice to block Fc receptors. Cells were washed and 100 µl of TCS or 2 µg/ml of purified mAbs were used to label surface molecules for 20 minutes on ice. Depending on the primary mAb used, 100 µl of a 1/100 dilution of FITC-conjugated goat-anti-rat or goat-anti-mouse was used as a secondary antibody. FL-HA was used at a concentration of 5 µg/ml. For double labeling, cells were labeled with 5 µg/ml of FL-HA and 2 µg/ml of PE-conjugated anti-CD14. Cells were analyzed on a FACScan® flow cytometer (Becton-Dickinson, Mississauga, ON) on Lysis II® software (Becton-Dickinson, Mississauga, ON). Size-selected PBMCs were gated on the basis of larger size (forward scatter; FSC) and increased granularity (side scatter; SSC). To select CD14 positive populations, cells were gated on the basis of their ability to bind to PE-conjugated anti-CD14 mAbs. To control for
variability in cytokine responses by donor PBMCs, each experiment was repeated at least 3 times with 3 different donors to confirm results.

2.23 Chlorate treatment of KG1a cells

KG1a cells at a concentration of $1 \times 10^6$ cells/ml in RPMI 1640 supplemented with 10% FCS were treated with increasing concentrations of sodium chlorate for 24 hours at 37°C, 5% CO$_2$ and HA binding was assessed by flow cytometry with 5 μg/ml of FL-HA as described in section 2.10. Sodium chlorate was dissolved in RPMI 1640 and added to KG1a cells at concentrations of 50, 100, 150, and 200 mM. Cell viability was tested by trypan blue exclusion whereby cells were resuspended in an equal volume of 0.05% trypan blue in PBS and live cells scored on a haemocytometer.
CHAPTER 3

Investigation of potential cytoskeletal associations of CD44

3.0 Rationale

As a molecule implicated in leukocyte extravasation and cell migration, CD44 is likely to be involved in the cytoskeletal reorganization required for cells to change their shape and migrate. Thus it is reasonable to hypothesize that CD44 associates with components of the cytoskeleton to facilitate cell locomotion. CD44 is abundantly expressed on a wide variety of cell types, including fibroblasts. However in fibroblasts, a significant proportion of CD44 remains detergent insoluble after Triton X-100 extraction. It has been thought that the insolubility of molecules after Triton X-100 extraction correlates with an association with the actin cytoskeleton. CD44 has been shown to interact either directly or indirectly with actin (119, 128, 145) and intermediate filaments (85). However, our laboratory has established that CD44 insolubility in NIH 3T3 cells cannot be attributed to a direct interaction with actin or with cytoskeletal molecules such as vimentin and spectrin as determined by immunofluorescence microscopy after treatments with agents that disrupt the cytoskeleton (205). However, another explanation is that these molecules localize to detergent insoluble fractions of cells, referred to as low density lipid domains, lipid rafts, detergent insoluble glycolipid enriched complexes (DIGs), or glycolipid enriched membrane (GEM) fractions. Both the GPI-anchored protein, Thy-1, and the calcium and phospholipid binding protein annexin II, were found in these complexes. There are a number of similarities between these proteins and CD44. One is the striking similarity in the staining patterns of Thy-1, annexin II, and CD44 upon immunofluorescence microscopy (60, 205, 206). Secondly, both GPI-anchored proteins and CD44 have a Triton X-100 insoluble component in fibroblasts. This, combined with reports documenting the migration of GPI-anchored proteins such as the folate receptor and annexin II in glycolipid enriched fractions (171, 172, 207), led to the hypothesis that CD44 could also migrate with low density material upon equilibrium density gradient centrifugation on sucrose. Here, I
wanted to investigate CD44 insolubility and examine if it could be attributed to migration with low density material on sucrose gradients. Furthermore, characterization of the region of CD44 responsible for its localization in the low density fraction was undertaken using CD44 chimeric molecules.

3.1 Triton X-100 insolubility of CD44 in NIH 3T3 cells

It had previously been shown that a fraction of CD44 in BHK cells and Swiss 3T3 fibroblasts was insoluble after Triton X-100 extraction (60, 85, 119, 145). By Western blot of whole cell lysates from NIH 3T3 cells, it was determined that the major form of CD44 expressed in these cells is a species of 85 kD which represents the standard form of CD44 (CD44H). It was observed that approximately 30-35% of CD44 in NIH 3T3 cells remained detergent insoluble after extraction with 1% Triton X-100 (Fig. 3.1A). The solubility of CD44 is not affected by the cell density of the NIH 3T3 cells, and by extension, whether the cells are in the log phase of growth or nearing confluency at the time of lysis.

Immunofluorescence microscopy has demonstrated that CD44 is evenly distributed on the surface of NIH 3T3 cells (Fig. 3.1B). Upon solubilization of NIH 3T3 cells with 0.5% Triton X-100, a unique lacy pattern of CD44 staining was observed (Fig. 3.1B) and a similar pattern was observed when the CD44 cytoplasmic deletion mutant was stained as well (205). This result indicated that insolubility could not be attributed to cytoskeletal associations mediated by the CD44 cytoplasmic domain. The lacy pattern was also very different from the pattern of actin stress fibres observed by phallolidin-TRITC staining (205), which led to the hypothesis that an association with detergent insoluble lipids rendered CD44 Triton X-100 insoluble and was responsible for the unique pattern of CD44 staining by immunofluorescence.

3.2 Migration of CD44 from NIH 3T3 cells with low density lipid fractions

To determine if CD44 was associated with lipids, the Triton X-100 lysate from NIH 3T3 cells was subjected to equilibrium density gradient centrifugation on a discontinuous sucrose
**Figure 3.1.** Triton X-100 extraction of CD44 from NIH 3T3 cells. (A) NIH 3T3 cells at different cell densities ($10^5$ cells/ml) were lysed in 1% Triton X-100 lysis buffer and the soluble fraction (S) separated from the insoluble pellet (P) by centrifugation at 12 000 g. CD44 was detected by western blotting with a 1/20 dilution of tissue culture supernatants (TCS) of the anti-CD44 mAb IM7.8.1 after electrophoresis under non-reducing conditions. The positions of prestained $M_r$ markers are indicated on the left in kilodaltons. (B) Expression of CD44 in NIH 3T3 cells before and after extraction with 0.5% Triton X-100 was detected using the anti-CD44 mAb IRAWB 14 at 1000X magnification on a Zeiss axiophot immunofluorescent microscope (scale bar 10 μM). NIH 3T3 cells labeled with FITC-conjugated goat-anti-rat Ig were the negative control.
gradient (204). This procedure separates lipid-rich components of the Triton X-100 insoluble material from the cytoskeleton and remnants of the nucleus (159). TCA precipitated proteins or CD44 immunoprecipitates from eight sucrose fractions plus the insoluble pellet were immunoblotted with anti-CD44 mAbs. The 85 kD standard form of CD44 was again observed to be the major isoform of CD44 expressed in NIH 3T3 cells (Fig. 3.2). Upon separation of cell lysates on sucrose gradients, detergent insoluble lipids migrate to fractions 3-4, the fractions 5-8 contain detergent soluble proteins, and the cytoskeletonally associated proteins migrate in the pellet at the bottom of the gradient (204). After lysis in Triton X-100, a nonionic detergent, CD44 was found in all three compartments. Approximately 28% of total CD44 expressed in NIH 3T3 cells migrated to the low density sucrose fractions, 65% of the CD44 protein was found to migrate in the soluble fractions, and in this experiment, 7% of the protein was in the Triton X-100 insoluble pellet (Fig. 3.2A). On average, approximately 18% ± 12% of CD44 in NIH 3T3 cells migrates in the low density sucrose fractions (n=10) and the remaining protein is found in the soluble fractions and pellet at the bottom of the gradient. If the gradient pellet was resuspended in sample buffer, run on an SDS-PAGE gel and immunoblotted with anti-CD44 mAbs, no CD44 reactivity was observed. However, if the pellet was boiled in 1% SDS and then diluted with 1% Triton X-100 lysis buffer and CD44 was immunoprecipitated, a CD44 reactive band was observed in the pellet. It may be that the volume of sample buffer used to resuspend the pellets for electrophoresis contained insufficient SDS to solubilize the cytoskeletonally associated proteins from the pellet. These data indicate that CD44 can be found in three distinct pools in NIH 3T3 cells. It was demonstrated that the insolubility of CD44 in Triton X-100 cannot be attributed wholely to an association with the actin cytoskeleton. Instead, the Triton X-100 insoluble pool of CD44 may consist of cytoskeletonally associated CD44, which would localize to the sucrose gradient pellet, and lipid associated CD44, which would migrate to the low density fractions of the gradient. Experiments detailing the presence of cytoskeletal markers in the pellet are described later.

To further characterize the lipid associated pool of CD44 in NIH 3T3 cells, sucrose gradients of cells lysed in another nonionic detergent, n-octyl-β-D-glucopyranoside (octyl
Figure 3.2. Equilibrium density gradient analysis of CD44 from NIH 3T3 cells. NIH 3T3 cells were lysed in 1% Triton X-100 (A) or 60 mM n-octyl-β-D-glucopyranoside (B) and centrifuged to equilibrium on a sucrose step gradient. CD44 was immunoprecipitated from sucrose fractions using the anti-CD44 mAb IM7.8.1 and separated on a 7.5% non-reducing SDS-PAGE gel. The gradient pellet was extracted with 100 µl of 10 mM Tris pH7.5, 1%SDS, boiled for 10 min and then diluted 10-fold with 1% Triton X-100 lysis buffer prior to immunoprecipitation of CD44. CD44 was detected on membranes using 2 µg/ml of biotinylated IM7.8.1 and HRP-conjugated streptavidin. IM7.8.1 conjugated to protein G sepharose was the negative control (C). The positions of prestained $M_r$ markers are indicated on the left in kilodaltons.
CD44

A
Fraction: C 1 2 3 4 5 6 7 8 P
175 ——
83 ——
62 ——
48 ——

Triton X-100

B
Fraction: C 1 2 3 4 5 6 7 8 P
175 ——
83 ——
62 ——
48 ——
n-Octyl-β-D-glucopyranoside
glucoside) were centrifuged to equilibrium. GPI-linked proteins, which can also be found in the Triton X-100 insoluble fraction of cells, are solubilized by octyl glucoside and the structure of this nonionic detergent is similar to that of glycolipids, which are a major component of so-called lipid rafts. It is possible that this structural similarity allows octyl glucoside to associate with lipids in the rafts, thereby solubilizing them and dissociating lipid-protein associations (159). Upon immunoprecipitation of CD44 and western blot, the major protein observed was 85 kD, consistent with the standard isoform of CD44 (Fig. 3.2B). In contrast to the 35% of CD44 which is Triton X-100 insoluble in NIH 3T3 cells, only 18% of total CD44 migrated in low density lipid fractions. In addition, octyl glucoside lysis completely solubilized CD44, as it was not observed in the octyl glucoside insoluble pellet. Approximately 82% of total CD44 was soluble in octyl glucoside and migrated in fractions 5-8, with the majority localizing to fractions 7 and 8. Thus octyl glucoside was able to solubilize some Triton X-100 insoluble CD44 and reduce the amount of CD44 that migrated to the low density lipid fractions. In fact the percentage of CD44 lost from the pellet fraction and fractions 3 and 4 of the Triton X-100 lysed cells (Fig. 3.2A), 19%, correlates exactly to the gain in the amount of CD44 in the octyl glucoside soluble fractions, indicating octyl glucoside can indeed solubilize Triton X-100 insoluble material, potentially by partially dissociating lipid-lipid and lipid-protein interactions. It is possible that at higher concentrations of octyl glucoside, localization of CD44 to the low density sucrose fraction would have been completely prevented.

3.3 Migration of other molecules expressed in NIH 3T3 cells to low density lipid fractions

To further characterize what other molecules may be present in the low density fraction of NIH 3T3 cells, fractions from sucrose gradients were immunoblotted with a variety of antibodies to examine if other proteins expressed in NIH 3T3 cells co-migrated with CD44 in the low density fractions. Three proteins were chosen for further analysis, the nonreceptor protein tyrosine kinase p60src (src), annexin II, a cytoskeletal linker protein, and actin. Besides being rich in glycolipids,
sphingolipids, and cholesterol, lipid rafts contain GPI-linked proteins, and a number of molecules involved in signal transduction (159, 171, 172). In fact, several src family kinases have been found to migrate in the buoyant fractions of sucrose gradients. Annexin II is a calcium and phospholipid binding protein which was cloned as a substrate for src, and which has been implicated in numerous physiological functions involving the membrane and calcium (reviewed in 208). However, the exact role of annexin II is poorly understood. Actin is an integral component of the actin cytoskeleton.

NIH 3T3 cells lysed in 1% Triton X-100 were centrifuged to equilibrium on a step gradient of sucrose (see Materials and Methods for details). Immunoblotting with a polyclonal antisera displaying reactivity against src and p60<sup>yes</sup> (yes) resulted in proteins migrating at approximately 60-62 kD, representing src and yes, and what was determined to be a non-specific protein band by others in the laboratory, at approximately 80 kD. The majority of the proteins (approximately 83%) localized in the buoyant fractions while the remaining 17% of src and yes expressed in NIH 3T3 cells was found in the soluble fractions 5-7 of sucrose (Fig. 3.3A). Only 10% of annexin II migrated to the low density fractions on sucrose gradients, with the remaining 90% of the protein localizing to the soluble fractions (Fig. 3.3B). Neither src, yes, nor annexin II was found in the Triton X-100 insoluble pellet at the bottom of the gradient. By contrast, immunoblotting with an anti-actin polyclonal mAb, C4 (ICN), revealed a very different pattern of migration on sucrose gradients compared to src, yes, and annexin II. The anti-actin mAb stained a protein species of approximately 45 kD in agreement with the predicted <i>M</i><sub>r</sub> for actin. Approximately 4% of actin was localized to the low density lipid fraction, another 16% of the protein migrated to the soluble fractions, and the majority of actin (approximately 80%) was in the pellet as expected (Fig. 3.3C), indicating that the pellet contained cytoskeletal elements. Bands at 80 kD and 60 kD in the actin blot were remnants from the previous immunoblot with the anti-src antibody since the membrane was not stripped prior to reprobing with the anti-actin mAb. Thus, the migration patterns of various different proteins in NIH 3T3 cells varies and may correlate with their function in the cell. Src family kinases, which are part of the signal transduction machinery of the cells, largely
Figure 3.3. Identification of other molecules in low density lipid fractions after equilibrium density gradient centrifugation of NIH 3T3 Triton X-100 lysates. Triton X-100 lysates from NIH 3T3 cells were centrifuged to equilibrium on sucrose and the protein from each fraction was precipitated with TCA, resuspended in sample buffer, and separated on a 7.5% reducing SDS-PAGE gel. Src and yes proteins (A) were detected using a 1/500 dilution of the anti-c-src polyclonal antisera, sc-18 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); annexin II (B) was detected using a 1/5000 dilution of the anti-annexin II polyclonal antibody (Transduction Laboratories, Lexington, KY); and actin (C) was detected using the C4 anti-actin mAb (ICN Biomedicals Inc., St. Laurent, PQ) at a 1/2000 dilution. The positions of prestained $M_r$ markers are indicated on the left in kilodaltons.
A

Fraction: 1 2 3 4 5 6 7 8 P

175 —
83 —
62 —
48 —
33 —

src/yes

B

Fraction: 1 2 3 4 5 6 7 8 P

175 —
83 —
62 —
48 —
33 —

annexin II

C

Fraction: 1 2 3 4 5 6 7 8 P

175 —
83 —
62 —
48 —
33 —

actin
localized to the low density lipid fractions, while actin, a major component of the actin cytoskeleton was mostly found in the gradient pellet. Actin found in the soluble fraction may be represented by actin monomers and it has been reported that globular actin (G-actin) migrates to lipid rafts (172). However, it should be noted that the co-migration of these proteins with low density material does not indicate that they directly associate with CD44. Rather these lipid fractions may act as subdomains that concentrate components of the cell that are important for certain cellular functions that remain poorly defined at this time.

3.4 Migration of CD44 from NIH 3T3 cells in the low density lipid fraction is mediated by the transmembrane domain of CD44

Given that a proportion of CD44 migrated with the low density lipid fraction in NIH 3T3 cells, it was of interest to determine what structural features of CD44 mediated the migration to these fractions. It has been reported that CD44 associates with cytoskeletal elements such as ankyrin (58, 148-150), and members of the ERM (ezrin, radixin, moesin) family of molecules (151-153) via its cytoplasmic domain which may be predicted to result in Triton X-100 insolubility. However, others have observed that deletion of the cytoplasmic domain of CD44 does not reduce the Triton X-100 insolubility of CD44 (60, 147, 205). To determine if the cytoplasmic tail of CD44 mediated migration of CD44 to low density lipid fractions, NIH 3T3 cells were transfected with a construct containing the CD44.1 allele lacking a cytoplasmic tail. NIH 3T3 cells express the CD44.2 allele endogenously but the cytoplasmic deletion mutant can be selectively immunoprecipitated using an the allele-specific mAb, RAMBM 44. Triton X-100 lysis and centrifugation of the transfected cells on sucrose gradients resulted in a band at 75 kD, consistent with deletion of the cytoplasmic domain of CD44. The same pattern of migration was observed for the mutant (Fig. 3.4) as for endogenous CD44 (Fig. 3.2A) upon analysis of the sucrose fractions. Approximately 17% of CD44 Δcytoplasmic tail was found to localize in the low density fractions 3 and 4. The remainder of the truncated CD44 migrated in the soluble fractions and a very small proportion (1%) was in the insoluble pellet (Fig. 3.4). Considering that on average, approximately
Figure 3.4. Equilibrium density gradient analysis of the CD44 cytoplasmic deletion mutant from NIH 3T3 cells. NIH 3T3 cells transfected with the CD44 Δcytoplasmic tail were lysed in 1% Triton X-100 and centrifuged to equilibrium on a sucrose step gradient. CD44 was immunoprecipitated from sucrose fractions using the anti-CD44.1 allele-specific mAb RAMBM 44 and separated on a 7.5% non-reducing SDS-PAGE gel. The gradient pellet was extracted with 100 μl of 10 mM Tris pH7.5, 1%SDS, boiled for 10 minutes and then diluted 10-fold with 1% Triton X-100 lysis buffer prior to immunoprecipitation of the CD44 Δcytoplasmic tail mutant. CD44 was detected on membranes using 2 μg/ml of biotinylated IM7.8.1 and HRP-conjugated streptavidin. RAMBM 44 conjugated to goat-anti-rat Ig sepharose was the negative control (C). The positions of prestained Mᵋ markers are indicated on the left in kilodaltons.
18% of the full length CD44 molecule migrated in the low density fraction, these data indicate that the cytoplasmic domain of CD44 is not required for migration to the low density lipid fractions of NIH 3T3 cells.

To test whether the CD44 transmembrane domain was responsible for mediating migration to low density lipid fractions, an L cell transfectant constructed by Perschl et al. was used (55). These L cells expressing the CD44.2 allele endogenously were transfected with the CD44.1 allele containing the transmembrane domain of CD45 (CD44-CD45 TM) and it was shown that this mutant was completely soluble in Triton X-100 (155). With that result in mind, these cells were lysed in Triton X-100 and centrifuged on sucrose gradients. As a control, untransfected L cells were also run on sucrose gradients to establish that a proportion of endogenous CD44 in L cells also migrated to the buoyant fractions of sucrose gradients. Indeed, upon immunoblotting the sucrose fractions with anti-CD44 mAbs, it was observed that a proportion of CD44 migrated into fractions 3 and 4 and represent approximately 36% of total CD44 in L cells (Fig. 3.5A). By contrast, the CD44-CD45 TM mutant did not migrate to the low density lipid fractions (Fig. 3.5B) and the majority of the protein (>98%) was found in soluble fractions 7 and 8. Both endogenous CD44 in L cells and the CD44-CD45 TM mutant had a $M_t$ of 85 kD on SDS-PAGE gels (Fig. 3.5). To try to further localize a particular region of the transmembrane domain as important for migration with lipids, the cysteine in the transmembrane domain was mutated to an alanine. This mutant made in the CD44.1 allele and transfected into NIH 3T3 cells, was still found in the low density fraction on sucrose gradients (data not shown), suggesting that this cysteine residue was not required for the migration of CD44 to the low density fractions. Cumulatively, these data demonstrate that the transmembrane domain but not the cytoplasmic domain of CD44 mediates its migration with the low density lipid fraction.
Figure 3.5. Requirement for the transmembrane domain of CD44 for migration to low density lipid fractions. Control L cells (A) and L cells transfected with CD44-CD45 TM (B) were lysed in 1% Triton X-100 and centrifuged to equilibrium on a sucrose step gradient. CD44 was immunoprecipitated from sucrose fractions using the anti-CD44 mAb IM7.8.1 in the case of control L cells and the RAMBM 44 CD44.1 allele-specific mAb in the case of the CD44-CD45 TM chimeric molecule. Immunoprecipitates were separated on 7.5% non-reducing SDS-PAGE gels and CD44 was detected on membranes using 2 μg/ml of biotinylated IM7.8.1 and HRP-conjugated streptavidin. The positions of prestained $M_r$ markers are indicated on the left in kilodaltons.
L cells

A

Fraction: 1 2 3 4 5 6 7 8 P

CD44

B

Fraction: 1 2 3 4 5 6 7 8 P

CD44 (CD45 TM)
CHAPTER 4

Analysis of CD44 interactions with hyaluronan in murine L cell fibroblasts

4.0 Rationale

The adhesive properties of CD44 are tightly regulated. Three different binding states have been identified for CD44 binding to HA: constitutive, inducible, and non-binding (100). Several factors can regulate the ability of CD44 to bind HA and many of these effects are cell-type and activation state specific. Both N- and O-linked glycosylation have been shown to influence the HA binding ability of CD44. The presence of N-linked oligosaccharides can have an inhibitory (116, 117) or enhancing (108, 110) effect on CD44-mediated HA binding. O-linked oligosaccharides either have no effect (117) or inhibit HA binding as well (111, 112). CD44 can also be modified by the addition of glycosaminoglycans (GAGs) such as chondroitin sulfate (CS), heparan sulfate (HS), and keratan sulfate (KS). KS modification on CD44 has been shown to have a negative effect on HA binding in a colon carcinoma cell line (109). Except for a few reports, the effect of GAG modification, especially HS and CS, on the HA binding ability of CD44 has not been very well examined and it is not clear which of these factors regulate HA binding in any one cell type. While approaches such as enzymatic digestion and the use of biosynthetic inhibitors can be used to assess the relative contribution of GAGs to HA binding, data generated by these methods can be hard to interpret due to the difficulty in determining the precise effects of the treatment on the cell surface GAG moieties and whether the complete removal of the GAGs was accomplished. An alternative approach was to take advantage of a series of L cell lines deficient in GAG synthesis to determine the effect of HS and CS on CD44 binding to hyaluronan (HA). These L cell lines were isolated on the basis of their resistance to herpes simplex virus-1 (HSV-1) infection. Gro2C cells are L cell variants deficient in the synthesis of HS. Selection of these gro2C cells for further resistance to HSV-1 infection gave rise to two L cell variants, sog9 and sog8, which are defective in synthesis of all GAGs. Sog8 cells also have additional defects in oligosaccharide processing.
By comparing the HA binding ability of CD44 in each variant cell line, the contribution of HS and CS in facilitating the functional interaction between CD44 and HA is determined. It will be demonstrated that CS plays an important role in the regulation of this interaction.

4.1 CD44 expression and HA binding ability of L cell variants

In this study, L cell lines defective in the synthesis of GAGs and in the addition of oligosaccharides were used to investigate the role of these post-translational modifications on CD44-HA binding (190, 191). Using highly sensitive anion exchange chromatography, it had been demonstrated previously that gro2C cells, derived from parental L cell fibroblasts, are defective in HS synthesis. Sog9 cells, derived from gro2C cells, contain an additional defect in the GAG synthesis pathway such that they have lost the ability to synthesize any sulfated GAGs. Sog8 cells are similar to sog9 cells except that they contain additional defects in the processing of N-linked and O-linked oligosaccharides. The characteristics of these cell lines are summarized in Table 4.1.

Table 4.1. Characteristics of L cell variants

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Parental line</th>
<th>Glycosaminoglycan synthesis</th>
<th>Glycosylation</th>
<th>HA binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>LM(tk)</td>
<td>--</td>
<td>+</td>
<td>Normal</td>
<td>Inducible</td>
</tr>
<tr>
<td>gro2C</td>
<td>LM(tk)</td>
<td>--</td>
<td>Normal</td>
<td>Inducible</td>
</tr>
<tr>
<td>sog9</td>
<td>gro2C</td>
<td>--</td>
<td>Normal</td>
<td>None</td>
</tr>
<tr>
<td>sog8</td>
<td>gro2C</td>
<td>--</td>
<td>Defective</td>
<td>Constitutive/inducible</td>
</tr>
</tbody>
</table>

a Determined by HPLC; b glycosylation assessed by pulse-chase labeling; c determined by FACS analysis using FL-HA; d(190); e(191); f(192) and this report

To assess the role of glycosaminoglycan synthesis on CD44-HA binding, fluoresceinated HA (FL-HA) was used to quantify the relative affinity of HA for each of the cell lines by flow cytometry (Fig. 4.1). The parental murine L cells did not constitutively bind FL-HA, but could
Figure 4.1. Surface expression of CD44 and HA binding ability of L cell variants. The expression of CD44 and the HA binding ability of L cells, gro2C, sog9, and sog8 cells was determined by flow cytometry. Expression levels of CD44 were determined using the anti-CD44 mAb IRAWB 14. Constitutive HA binding ability was examined using 15 μg/ml of fluoresceinated HA (FL-HA) and inducible HA binding was determined after pretreatment with the IRAWB 14 mAb. Unlabeled L cells were the negative control.
FL-HA + IRAWB 14
Negative control  L cells  gro2C  sog9  sog8

FL-HA

Anti-CD44 antibody

Fluorescence intensity

Cell number
be induced to bind FL-HA after pretreatment with the IRAWB 14 mAb. This mAb binds to the extracellular domain of CD44 and increases CD44-mediated HA binding by inducing a conformational change in CD44 and/or aggregation of CD44 on the cell surface (15). To determine whether HS or CS synthesis was required for inducible binding, FL-HA binding was examined on gro2C and sog9 cells. IRAWB 14-inducible binding was observed in the gro2C cells, indicating that HS is not required for HA binding to these cells. By contrast, sog9 cells could not be induced to bind FL-HA, suggesting that CS plays a role in the CD44-HA interaction. Cell surface CD44 expression on all of the L cell variants was similar as determined by flow cytometry (Fig 4.1). Taken together, these results indicate that alterations in CS synthesis can have a demonstrable effect on the interaction of HA with CD44. Interestingly, sog8 cells bound FL-HA constitutively and could be further induced to bind increasing amounts of FL-HA following incubation with IRAWB 14. This result suggested that CS was no longer required to induce HA binding when N-linked and O-linked oligosaccharides were incompletely processed.

To verify that the observed inducible and constitutive FL-HA binding was CD44-mediated, L and sog8 cells were incubated with the anti-CD44 mAb, KM201 (Fig. 4.2). KM201 was able to block the FL-HA binding on both IRAWB 14 induced L cells and sog8 cell, indicating that all FL-HA binding was mediated by CD44.

4.2 Molecular characteristics of CD44 expressed on L cells

The forms of CD44 expressed in each cell line were analyzed to determine what effect the GAG synthesis and glycoprotein processing defects had on CD44. CD44 in L cells has previously been shown to be a protein of relative molecular mass of 85 kD and these cells do not express other isoforms of CD44. This form is the standard form of CD44, CD44H, which contains none of the alternatively spliced exons (117). To characterize the forms of CD44 expressed in the control L, gro2C, sog9 and sog8 cells, whole cell lysates were immunoblotted with the anti-CD44 mAb KM201 (Fig 4.3). By this method, it was demonstrated that L, gro2C, and sog9 cells expressed a major 85 kD form of CD44, CD44H, with no higher $M_r$ isoforms detected. By contrast, the
Figure 4.2. Inducible HA binding is CD44-mediated. L cells and sog8 cells were incubated in the presence and absence of the inducing mAb, IRAWB 14, and FL-HA binding was then assessed. The anti-CD44 mAb KM201 was used to block CD44-dependent HA binding.
L cells

sog8

Cell number

Fluorescence intensity

10^1 10^2 10^3

10^1 10^2 10^3

Negative control
FL-HA
FL-HA + KM201
FL-HA + IRAWB14
FL-HA + IRAWB14 + KM201
Figure 4.3. Relative molecular mass of CD44 expressed in the L cell variants. A western blot of CD44 from cell lysates of the L cell lines using the anti-CD44 mAb KM201 at a 1/20 dilution is shown (see Materials and Methods for details). The positions of prestained $M_r$ markers are indicated on the left in kilodaltons.
apparent $M_r$ of CD44 expressed in sog8 cells was approximately 70 kD. This reduction in apparent $M_r$ is consistent with the presence of a defect in the glycoprotein processing pathway of sog8 cells.

When CD44 was immunoprecipitated from L cells after radioactively labeling with $[\text{35}S]\text{sulfate}$, the 85 kD form of CD44, CD44H, and a higher $M_r$ species of approximately 175 kD was observed (Fig. 4.4). This higher $M_r$ species was immunoprecipitated using conditions that would eliminate co-precipitation of weakly associated CD44 proteins (Fig. 4.4, lane 1), indicating that is was likely to be a sulfated higher $M_r$ form of CD44. To verify this, CD44 immunoprecipitates were boiled in SDS to separate all non-covalently associated proteins and then re-precipitated. Although less total protein was re-immunoprecipitated, approximately equal ratios of the higher and lower $M_r$ forms were present (Fig. 4.4, lane 3), indicating that this was a sulfated higher $M_r$ form of CD44.

To identify the sulfated moiety on the larger form of CD44, the CD44 immunoprecipitate was treated with chondroitin ABC lyase (Fig. 4.4, lane 4). The 175 kD band was sensitive to digestion with this enzyme, indicating that the higher $M_r$ form of CD44 was modified by CS. As expected, immunoprecipitation of CD44 from sog9 cells, which are deficient in the synthesis of CS, produced only the lower $M_r$ sulfated species (Fig. 4.4, lane 5).

4.3 Characterization of the role of chondroitin sulfate in IRAWB 14-inducible HA binding

The role of sulfation in CD44-HA interactions was investigated by treatment with sodium chlorate. Sodium chlorate is a potent inhibitor of the sulfation of proteins and GAGs (209, 210). Because chondroitin containing GAGs are subsequently modified by sulfate addition, it was important to determine the relative importance of these moieties. Time-course and dose-response studies were performed, which indicated that 10 mM sodium chlorate treatment for 2 days was the optimal concentration to induce an effect without causing a significant decrease in cell viability. L cells grown in the presence of 10 mM sodium chlorate lost IRAWB 14 inducible binding (Fig. 4.5), whereas sog8 cells grown in the same manner were relatively unaffected. Thus, it appears
Fig. 4.4. Relative molecular mass of CD44 after $[^{35}\text{S}]$sulfate labeling of L cell variants. Equivalent amounts of CD44 from $[^{35}\text{S}]$sulfate labeled L cells were immunoprecipitated from L cells before (-, lane 2) and after (+, lane 4) treatment with chondroitin ABC lyase. Sog9 cells are shown in lane 5. Cells were lysed in RIPA buffer (lane 1) or 1% Triton X-100 lysis buffer (lanes 2-5). CD44 was re-immunoprecipitated from a CD44 immunoprecipitate that had been denatured in 1% SDS (lane 3). A control immunoprecipitate in the absence of the immunoprecipitating mAb IM7.8.1 is indicated in lane C. The positions of prestained $M_r$ markers are indicated on the left in kilodaltons.
Figure 4.5. HA binding ability after treatment of cells with sodium chlorate. L cells, sog8, and NIH 3T3 cells were incubated in the presence of 10 mM sodium chlorate for 2 days and then tested for the ability to bind to 15 μg/ml of FL-HA either constitutively or after IRAWB 14 induction as determined by flow cytometry.
The figure shows histograms for cell number and fluorescence intensity for L cells, sog8, and NIH 3T3 cells under different conditions.

- **L cells**:
  - Negative control
  - FL-HA

- **sog8**:
  - FL-HA + 10 mM sodium chlorate
  - FL-HA + IRAWB 14 + 10 mM sodium chlorate

- **NIH 3T3**:
  - Negative control
  - FL-HA
  - FL-HA + 10 mM sodium chlorate
that sulfation is important for inducible CD44-HA interactions in L cells. The lack of effect of this inhibitor on sog8 cells suggests that it is the sulfation of chondroitin that is required for inducible binding.

To determine whether sulfation affects inducible HA binding in other cells, similar studies were performed with sodium chlorate on NIH 3T3 cells (Fig. 4.5). It is clear from these data that IRAWB 14-inducible binding was inhibited in this cell line, which indicates that sulfation is an important post-translational modification that facilitates functional CD44-HA interactions in different cells.

4.4 Characterization of CD44 in sog8 cells

Since the glycosylation defect in sog8 cells dramatically altered the binding profile of FL-HA to CD44, pulse-chase experiments with $[^{35}S]$methionine and $[^{35}S]$cysteine were performed to characterize CD44 in sog8 cells (Fig. 4.6). After a 20 minute pulse, newly synthesized CD44 had the same apparent $M_t$ (approximately 62 kD) in both L cells and sog8 cells. This species was sensitive to digestion by endoH, indicating that protein translation and initial processing in these cells were normal. Although CD44 became endoH resistant in both cell lines at the same rate, the mature form present on sog8 cells was significantly smaller (70 kD) compared to the normal form of 85 kD present on L cells (Fig. 4.6). This indicates that initial processing of N-linked sugars occurs in sog8 cells, resulting in the addition of N-acetyl-glucosamine, but that there is a serious alteration in the processing of oligosaccharide moieties after they reach the medial Golgi.

In other experiments performed by a collaborator, L. E. Esford, CD44 immunoprecipitates were treated with PNGaseF and O-glycosidase to investigate whether both N- and O-linked oligosaccharide processing of CD44 was defective in sog8 cells. PNGaseF treatment of CD44 immunoprecipitated from sog8 cells resulted in a smaller reduction in apparent $M_t$ (approximately 5 kD less) compared to the difference observed from CD44 isolated from L cells (192). This indicates that sog8 cells possess incompletely processed N-linked sugars. O-glycosidase treatment of CD44 from sog8 cells indicated a severe defect in the addition of O-linked oligosaccharides.
Figure 4.6. Pulse-chase analysis of [\textsuperscript{35}S]methionine and [\textsuperscript{35}S]cysteine-labeled CD44 from L cells and sog8 cells. CD44 immunoprecipitates from L cells (A) or sog8 cells (B) were treated with (+) or without (-) endoH after a 20 minute pulse of radiolabel (time 0) and the prescribed chase times (15, 30, 60, and 120 minutes) in unlabeled media. The positions of prestained $M_r$ markers are indicated on the left in kilodaltons.
A  

L cells

<table>
<thead>
<tr>
<th>EndoH:</th>
<th>(-)</th>
<th>(+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (minutes):</td>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>15</td>
</tr>
</tbody>
</table>

B  

sog8

<table>
<thead>
<tr>
<th>EndoH:</th>
<th>(-)</th>
<th>(+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (minutes):</td>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>15</td>
</tr>
</tbody>
</table>
Taken together, these results demonstrate that N-linked processing and the addition of O-linked oligosaccharides to CD44 are defective in the sog8 cells. This loss of fully processed oligosaccharides clearly alters the requirement for CS in CD44-mediated inducible HA binding by these cells. Thus, it has been demonstrated that although CS synthesis is required for IRAWB 14-inducible binding in L cells, it is not essential for binding when certain oligosaccharide moieties are absent.
CHAPTER 5

Role of sulfation in the induction of CD44-mediated leukocyte adhesion by pro-inflammatory soluble factors

5.0 Rationale

Regulation of cell adhesion is important for immune system function. CD44 is a tightly regulated cell adhesion molecule present on leukocytes and implicated in their rolling on endothelium during an inflammatory immune response (20). CD44 is normally present on leukocytes in an inactive state that cannot bind HA but can be converted to an active state by activation by antigen or cytokines (reviewed in 52, 211). While cytokine-mediated upregulation of CD44 expression and adhesion to HA has been demonstrated on B cells by IL-5 (28) and on airway smooth muscle cells by TNFα (212), at the time these experiments were initiated, few soluble factors were identified as capable of inducing HA binding on myeloid cells. Considering the role that cells of the myeloid lineage play in the production of an inflammatory response, I set out to identify some of these factors. In cases where activation of CD44 adhesion by antigen or cytokines has been documented, the molecular mechanism for conversion from an inactive CD44 molecule to an HA binding molecule is not well understood. The HA binding ability of CD44 has been shown to be affected by three types of post-translational modification: N-linked glycosylation, O-linked glycosylation and GAG addition (51, 52). Here, it is shown that sulfation is an additional post-translational mechanism that can convert inactive CD44 to its active, adhesive form and that this mechanism is induced by the pro-inflammatory cytokine tumour necrosis factor-α (TNFα) on SR91 cells. In addition, pro-inflammatory mediators such as TNFα, IL-1β, and LPS and monocyte-specific inflammatory chemokines such as MIP-1α and MCP-1 were tested for their ability to activate CD44-mediated HA binding on monocytes and their dependence on sulfation to mediate this induction.
5.1 Characterization of cell surface molecules expressed on SR91 leukocytes and SVEC4-10 endothelial cells

The cell lines used in this study, the SR91 leukocytes and the SVEC4-10 endothelial cells, were characterized with respect to the expression of cell surface molecules. The human leukemic cell line SR91 used in this study was derived from a patient at the B.C. Cancer Agency with acute lymphocytic leukemia. SR91 cells are characteristic of a myeloid progenitor cell type in that they express the myeloid-specific marker CD33 but are negative for the monocyte marker, CD14 and the lymphoid markers CD2 and CD7 (193). In addition, SR91 cells do not express markers for B cells; CD19 or CD20, nor do they express T lymphocyte specific markers; CD3, CD4, or CD8. However, they are positive for CD45, which is expressed on all hematopoietic cells (213). While Klingemann et al. reported that 25% of SR91 cells were positive for CD34, reflecting the progenitor characteristic of the line, I did not observe any CD34 expression on the cell surface as determined by flow cytometry (Fig. 5.1). With respect to cell adhesion molecules, SR91 cells express very little or no ICAM-1 and VCAM-1 (Fig. 5.1 and 214) and are positive for the expression of ICAM-2, ICAM-3, LFA-1 (214), CD44, CD43, and β1 integrins (Fig. 5.1).

SVEC4-10 cells are an endothelial cell line derived from lymph node stroma which were transformed by transfection with SV40. These cells retain the morphological and functional characteristics of normal endothelial cells (194). Staining of these cells with mAbs to cell adhesion molecules revealed that they do not express ICAM-1 (Fig. 5.2), ICAM-2, VCAM-1, or PECAM-1 on their cell surface (215). The lack of ICAM-1 and ICAM-2 expression on SVEC4-10 cells was confirmed by cell surface biotinylation and immunoprecipitation (215). However, SVEC4-10 cells do express surface CD44 which binds some FL-HA and can be further induced by IRAWB 14 to bind more FL-HA (Fig 5.2). This FL-HA binding is mediated by CD44 since it can be blocked by pre-incubation with the anti-CD44 mAb, KM201, which blocks the HA binding site of CD44 (196). In addition, they do not express Thy-1 on the cell surface (Fig. 5.2).
Figure 5.1. Characterization of cell surface molecules expressed on SR91 cells. The expression of various cell adhesion molecules on unstimulated and TNFα-treated SR91 cells was determined by flow cytometry. Expression levels of CD44, CD43, and CD34 were determined using the anti-CD44 mAb 3G12, the anti-CD43 mAb H5H5, and the anti-CD34 mAb 8G12, respectively. mAbs used to label ICAM-1, β1 integrin, and VCAM-1 were MEM 111, P5D2, and MCA907 respectively. Exon v10 containing CD44 isoforms were detected with the anti-CD44 mAb 2G1, labeled CD44R1, and the activation epitope of CD44 was labeled using the anti-CD44 mAb 7F4. FL-HA at a concentration of 5µg/ml was used to determine HA binding ability. Unlabeled SR91 cells were the negative control.
Figure 5.2. Characterization of surface molecules expressed on the murine SVEC4-10 endothelial cell line. Expression levels of CD44, ICAM-1, and Thy-1 were determined by flow cytometry with the anti-CD44 mAb IM7.8.1, the anti-ICAM-1 mAb YN1/1.7.4, and the anti-Thy-1 mAb T24/37.1. Constitutive HA binding ability was examined using 5 μg/ml of FL-HA and inducible HA binding was determined after pretreatment with the anti-CD44 mAb IRAWB 14. The anti-CD44 mAb KM201 was used to block CD44-dependent HA binding and unlabeled SVEC4-10 cells were the negative control.
5.2 Induction of CD44-mediated HA binding on SR91 cells by TNFα

During an inflammatory response, cytokines, including TNFα, stimulate leukocyte-endothelial interactions by the activation and upregulation of a variety of cell adhesion molecules (8). It had been observed that TNFα could induce the activation of LFA-1, β1 integrins and upregulate the expression of CD44 on SR91 cells (216). Thus, this cell line was used to investigate the effect of TNFα on the adhesive state of CD44.

Treatment of the SR91 cells with 10 ng/ml of TNFα for 24 hours resulted in an increase in the surface expression of the cell adhesion molecules ICAM-1 and CD44 (Fig. 5.1 and 5.3). In addition, TNFα also induced the binding of FL-HA by the SR91 cells (Fig. 5.3). FL-HA binding could be inhibited by the addition of anti-CD44 mAbs IM7.8.1, and 3G12, as well as by 50 μg/ml of unlabeled HA, indicating a specific interaction between CD44 and its ligand, HA (Fig. 5.3). Stimulation of SR91 cells with 500 U/ml of interferon-γ (IFNγ) also induced ICAM-1 expression but did not cause a significant increase in CD44 expression and did not induce FL-HA binding (Fig. 5.3), demonstrating the selectivity of the TNFα response on CD44.

Treatment of SR91 cells with TNFα also upregulated the expression of VCAM-1 significantly, and β1 integrins slightly (Fig. 5.1). TNFα could induce reactivity with the anti-CD44 mAb 7F4 (Fig. 5.1) as observed previously (214). 7F4 is thought to recognize an activation epitope of CD44 that appears to correlate with FL-HA binding. Finally TNFα stimulation caused an upregulation of alternatively spliced isoforms on the SR91 cells as determined by staining with the anti-CD44 mAb 2G1 (Fig. 5.1), which recognizes an epitope on variant exon 10 (197). Thus, TNFα can upregulate the expression of other cell adhesion molecules on SR91 cells and specifically, part of the increased CD44 expression can be attributed to an increase in the expression of variant CD44 (CD44v) isoforms containing variant exon 10.

5.3 Induction of CD44-mediated HA binding by pro-inflammatory cytokines and chemokines in SR91 cells

The SR91 cell line was used to investigate the effect of soluble factors on the adhesive state
Figure 5.3. Surface expression of CD44, ICAM-1, and HA binding ability of SR91 cells. The expression of CD44, ICAM-1 and the HA binding ability on untreated, TNFα-treated, or IFNγ-treated SR91 cells was determined by flow cytometry. Expression levels of CD44 and ICAM-1 were determined using the anti-CD44 mAb IM7.8.1 and the anti-ICAM-1 mAb MEM 111. Cells were preincubated with the anti-CD44 mAb IM7.8.1, or with unlabeled HA to block FL-HA binding. FL-HA was used at a concentration of 5 µg/ml. Unlabeled SR91 cells were the negative control.
of CD44. Treatment of the SR91 cells with 1 μg/ml of purified bacterial LPS for 24 hours resulted in a marginal increase in CD44 expression on the cell surface but no induction of FL-HA binding (Fig. 5.4). This result is not surprising in light of the fact that SR91 cells are negative for the expression of CD14, the receptor for bacterial LPS (193). Treatment of SR91 cells with 10 ng/ml of IL-1β for 24 hours resulted in a slight increase in the surface expression of CD44 but also did not induce FL-HA binding (Fig. 5.4). In contrast, as observed previously, TNFα at a concentration of 10 ng/ml caused an increase in CD44 expression and induced FL-HA binding in the SR91 cells (Fig. 5.4). Similar to LPS and IL-1β, both monocyte-activating chemokines, MIP-1α and MCP-1 also caused a slight increase in surface CD44 expression but were unable to induce FL-HA binding (Fig. 5.4). Taken together, these results demonstrate that none of the cytokines or chemokines tested that activate monocyte function during an inflammatory response affect CD44 adhesion with the exception of TNFα on SR91 cells. One explanation for this fact may be that SR91 cells are not primed to express the appropriate receptors for these pro-inflammatory mediators.

5.4 TNFα induced sulfation of CD44 on SR91 cells

Previous work in fibroblasts had suggested a role for sulfation in regulating the HA binding ability of CD44 (Chapter 4 and ref 217). To elucidate the molecular mechanisms by which TNFα induces the adhesive ability of CD44 on SR91 cells, I first determined which forms of CD44 were present and whether TNFα altered the sulfation pattern of CD44. Only the 85 kD standard form of CD44 (CD44H) was detected in the SR91 cells after immunoprecipitation and blotting with the anti-CD44 mAb IM7.8.1, and this form incorporated scant amounts of [35S]sulfate (Fig. 5.5). However, after TNFα stimulation, the sulfation was increased significantly, whereas CD44 expression was only slightly enhanced (Fig. 5.5). On average, sulfation of CD44 was increased 4.5 ± 2.4 times (n = 4), whereas CD44 expression increased by only 1.9 ± 0.4 times (n = 4). Unlike TNFα stimulation, IFNγ had no effect on sulfate incorporation into CD44. Although IFNγ was able to upregulate CD44 expression in this
Figure 5.4. Effect of pro-inflammatory cytokines and chemokines on HA binding ability on SR91 cells. The expression of CD44 and the HA binding ability on untreated, or SR91 cells treated with TNFα and IL-1β (10 ng/ml), LPS (1 µg/ml), and MIP-1α and MCP-1 (50 ng/ml) for 24 hours was determined by flow cytometry. Expression levels of CD44 and HA binding ability were determined using the anti-CD44 mAb IM7.8.1 and 5 µg/ml of FL-HA respectively. Unlabeled SR91 cells were the negative control.
Figure 5.5. [\(^{35}\text{S}\)]sulfate labeling of SR91 cells. SR91 cells (5 X 10^6), either unstimulated (-) or stimulated (+) with TNF\(\alpha\) or IFN\(\gamma\) were labeled with [\(^{35}\text{S}\)]sulfate. One quarter of the IM7.8.1-immunoprecipitated CD44 was subjected to SDS-PAGE under non-reducing conditions, then immunoblotted with 2 \(\mu\)g/ml of biotinylated anti-CD44 mAb IM7.8.1 followed by HRP-conjugated streptavidin. The remainder was resolved by SDS-PAGE under reducing conditions and exposed for autoradiography. The positions of prestained \(M_r\) markers are indicated on the left in kilodaltons.
experiment, this was not found to be significantly above the unstimulated value. Thus the sulfation of CD44 was induced by TNF\(\alpha\), not IFN\(\gamma\).

5.5 TNF\(\alpha\)-inducible HA binding on SR91 cells requires the sulfation of CD44

To determine whether the sulfation of CD44 was required for the induced adhesion of CD44 to HA, SR91 cells were incubated during the TNF\(\alpha\) stimulation period with 50 mM sodium chlorate, a potent sulfo-transferase inhibitor (209, 210). CD44-mediated FL-HA binding induced by TNF\(\alpha\) was abrogated in the presence of sodium chlorate (Fig. 5.6) as determined by flow cytometry. Chlorate treatment of cells also blocked the incorporation of \[^{35}S\]sulfate into CD44 (Fig. 5.7). However, chlorate treatment of SR91 cells did not prevent the increase in CD44 expression in response to TNF\(\alpha\) (Fig. 5.6 and Fig. 5.7) and did not affect cell viability, as assessed by trypan blue exclusion. This result indicated that an increase in CD44 expression alone could not account for the induction of CD44 adhesiveness. Thus, TNF\(\alpha\) stimulates the sulfation of CD44 leading to a conversion from an inactive form of CD44 to an active, HA binding form of CD44.

5.6 TNF\(\alpha\) induction of CD44-mediated leukocyte-endothelial cell interactions is sulfation dependent

Given that TNF\(\alpha\) promotes leukocyte adhesion and migration at inflammatory sites, I wanted to determine whether TNF\(\alpha\) could induce the adhesion of SR91 cells to endothelial cells. SR91 cells labeled with a fluoresceinated viable dye (CellTracker™ Green; CMFDA) were added to an endothelial cell monolayer in a static binding assay (see Materials and Methods for details). TNF\(\alpha\) stimulated SR91 cells, but not unstimulated or IFN\(\gamma\) treated cells, bound to the SVEC4-10 murine endothelial cell monolayer (Fig. 5.8). The TNF\(\alpha\) induced adhesion was not dependent on the upregulation of ICAM-1 expression as it was not inhibited by an anti-ICAM-1 mAb (Fig. 5.8B).
Figure 5.6. Effect of chlorate on HA binding ability of CD44 on SR91 cells.

Unstimulated and TNFα-stimulated SR91 cells were incubated in the presence of 50 mM sodium chlorate for 24 hours. CD44 (using the IM7.8.1 mAb) and FL-HA binding profiles were determined by flow cytometry. FL-HA was used at a concentration of 5 μg/ml. Unlabeled SR91 cells were the negative control.
Figure 5.7. Effect of chlorate on sulfation of CD44 on SR91 cells. $5 \times 10^6$ unstimulated (−) and TNFα-stimulated (+) SR91 cells were incubated in the presence (+) and absence (−) of 50 mM sodium chlorate and $[35\text{S}]$sulfate labeled. One quarter of the IM7.8.1-immunoprecipitated CD44 was subjected to SDS-PAGE under non-reducing conditions, then immunoblotted with 2 μg/ml of biotinylated anti-CD44 mAb IM7.8.1. The remainder was resolved by SDS-PAGE under reducing conditions and exposed for autoradiography. The positions of prestained $M_r$ markers are indicated on the left in kilodaltons.
Figure 5.8. Adhesion of SR91 cells to an SVEC4-10 endothelial cell monolayer. (A) Binding of unstimulated or TNFα-stimulated SR91 cells to a monolayer of SVEC4-10 cells (scale bar, 100 μm). (B) Percentage of $3 \times 10^5$ CMFDA-labeled SR91 cells binding to the SVEC4-10 monolayer, untreated or treated with TNFα, IFNγ, or TNFα and anti-ICAM-1 mAb MEM 111, as indicated. Percentage of cells binding was determined by fluorimetry.
A

SR91  TNFα

B

Binding (%)

0  10  20  30  40  50  60

-  TNFα  IFNγ  TNFα anti-ICAM-1
Preincubation of the TNFα treated SR91 cells with the anti-CD44 mAb IM7.8.1 prevented the interaction between SR91 cells and SVEC4-10 cells, indicating that the interaction was CD44-dependent (Fig. 5.9). Preincubation of the murine SVEC4-10 monolayer with a murine-specific CD44 mAb, KM201, also prevented TNFα stimulated SR91 cells from binding to the endothelial cells, inferring a homotypic, CD44-CD44 interaction. Both CD44 antibodies block HA binding in human and murine cells, respectively, which suggests that the HA binding regions of CD44 are involved in the interaction (Fig. 5.9). Preincubation of either the leukocytic SR91 cells or the endothelial SVEC4-10 cells with exogenous HA had no significant inhibitory effect on adhesion (Fig. 5.9). However, pretreatment of either cell line with hyaluronidase abolished the interaction, suggesting a role for endogenous HA in bridging the CD44-CD44 interaction (Fig. 5.9).

To determine if sulfation was required for the TNFα induced interaction between SR91 cells and SVEC4-10 cells, SR91 leukocytes were treated with the sulfation inhibitor sodium chlorate. Treatment of the SR91 cells with TNFα in the presence of 50 mM sodium chlorate prevented the binding of SR91 cells to the SVEC4-10 endothelial cell monolayer (Fig. 5.10). Thus, TNFα stimulates the sulfation of CD44 on SR91 cells which activates the adhesive function of CD44 such that it can bind the extracellular matrix component HA and mediate leukocyte-endothelial cell adhesion.

5.7 Characterization of the TNFα induced sulfation of CD44 on SR91 cells

Given that TNFα induced adhesion by CD44 was sulfation dependent, it became important to address the question of where sulfate moieties were incorporated on the CD44 molecule. CD44 can be decorated by a variety of post-translational modifications (reviewed in 50, 51, 52). Among the post-translational modifications that can also incorporate sulfate are the GAGs, N-linked and O-linked oligosaccharides, and finally, the protein backbone of CD44 can be sulfated on tyrosines. These modifications are therefore targets for further investigation. The approach taken was to remove $[^{35}S]$sulfate incorporated into CD44 during the TNFα stimulation period by enzymatic or chemical digestion.
Figure 5.9. Adhesion of SR91 cells to an SVEC4-10 endothelial cell monolayer is CD44-dependent. The percentage of $3 \times 10^5$ CMFDA-labeled SR91 cells binding to the SVEC4-10 monolayer, untreated or pretreated as indicated, was determined by fluorimetry. Pretreatment of SR91 cells or of SVEC4-10 cells was as indicated. Ab, anti-CD44 mAb (IM7.8.1 for SR91, KM201 for SVEC4-10), HA, hyaluronan; H, hyaluronidase.
Figure 5.10. Adhesion of SR91 cells to an SVEC4-10 endothelial cell monolayer is sulfation dependent. The percentage of $3 \times 10^5$ CMFDA-labeled SR91 cells binding to the SVEC4-10 monolayer, untreated, treated with TNF$\alpha$, or TNF$\alpha$ and 50 mM chlorate was determined by fluorimetry.
Heparitinase II treatment of CD44 immunoprecipitates from [\(^{35}\)S]sulfate labeled TNF\(\alpha\) stimulated SR91 cells to remove HS did not result in a loss of [\(^{35}\)S]sulfate label from CD44 (Fig. 5.11). Likewise, treatment of CD44 immunoprecipitates with keratanase or a combination of keratanase and keratanase II did not remove any [\(^{35}\)S]sulfate label (Fig. 5.11), indicating that TNF\(\alpha\) does not induce the sulfation of CD44 on heparan sulfate or keratan sulfate. As expected, hyaluronidase treatment of CD44 did not remove any [\(^{35}\)S]sulfate label (Fig. 5.12). Finally, treatment of the [\(^{35}\)S]sulfate labeled immunoprecipitates with chondroitin ABC lyase which cleaves chondroitin sulfate (CS) from the protein backbone also did not remove significant amounts of [\(^{35}\)S]sulfate (Fig. 5.13). Taken together, these data indicate that CD44 is not inducibly sulfated on GAGs upon TNF\(\alpha\) stimulation of SR91 cells and that GAG sulfation is not responsible for the induction of HA binding or endothelial cell binding observed in TNF\(\alpha\) treated SR91 cells.

To address whether CD44 was inducibly sulfated on oligosaccharides, CD44 immunoprecipitates from [\(^{35}\)S]sulfate labeled, TNF\(\alpha\) stimulated SR91 cells were digested with PNGaseF or O-glycosidase to remove N-linked and O-linked oligosaccharide sidechains respectively. Treatment of CD44 with PNGaseF did not remove any [\(^{35}\)S]sulfate label from the immunoprecipitated material although there was a reduction in the \(M_r\) of CD44 from 85 kD to approximately 70 kD, indicating that the enzyme was effective in cleaving N-linked sugars from the protein backbone (Fig. 5.13). Treatment of the CD44 immunoprecipitate with O-glycosidase also did not remove any [\(^{35}\)S]sulfate label, however, a reduction in \(M_r\) was not observed (Fig. 5.13). It has been documented that O-glycosidase can be refractory to cleaving complex, branched, O-linked mucin sidechains (technical info; Roche Diagnostics, Laval, PQ) and it is possible that this is the case for CD44 in TNF\(\alpha\) stimulated SR91 cells. Results from digestion of CD44 with PNGaseF and O-glycosidase were similar to those from CD44 digested with PNGaseF alone, adding weight to the argument that CD44 was refractory to digestion by O-glycosidase (Fig. 5.13).

Another approach was taken to address whether CD44 was inducibly sulfated on O-linked oligosaccharides. CD44 immunoprecipitates were treated with sodium hydroxide in a process
Figure 5.11. Removal of $[^{35}S]$sulfate on CD44 by glycosaminoglycan digestion. CD44 was immunoprecipitated from $5 \times 10^6$ $[^{35}S]$sulfate labeled SR91 cells stimulated with TNFα for 24 hours. Immunoprecipitates were subjected to digestion with keratanase (K), keratanase and keratanase II (K$^2$), heparitinase II (H), or left undigested (−). To quantify amounts, CD44 was immunoprecipitated from TNFα stimulated SR91 cells metabolically labeled with $[^{35}S]$methionine and $[^{35}S]$cysteine (see Materials and Methods for details). A control immunoprecipitate in the absence of the immunoprecipitating mAb IM7.8.1 is indicated in lane C. The positions of prestained $M_r$ markers are indicated on the left in kilodaltons.
SR91  |  TNFα
---|---
-  |  -  |  K  |  H  |  -  |  K  |  H  \\
|  175 |  83 |  62 |  48 |  33 |

[35S]sulfate  

[35S]sulfate

[35S]met/cys
Figure 5.12. Removal of $[^{35}\text{S}]$sulfate on CD44 by digestion of hyaluronan. CD44 was immunoprecipitated from $5 \times 10^6$ $[^{35}\text{S}]$sulfate labeled SR91 cells stimulated with TNF$\alpha$ for 24 hours and immunoprecipitates were subjected to digestion with hyaluronidase (Hy) or left untreated (-). The positions of prestained $M_r$ markers are indicated on the left in kilodaltons.
Figure 5.13. Removal of $[^{35}S]\text{sulfate}$ on CD44 by chondroitin sulfate and oligosaccharide digestion. CD44 was immunoprecipitated from $5 \times 10^6$ $[^{35}S]\text{sulfate}$ labeled SR91 cells stimulated with TNFα for 24 hours. Immunoprecipitates were subjected to digestion with chondroitin ABC lyase (Ch), PNGaseF (P), O-glycosidase (O), PNGaseF and O-glycosidase (P+O), or β-eliminated with NaOH (β) (see Materials and Methods for details). To quantify protein amounts, $[^{35}S]\text{sulfate}$ labeled SR91 cells were surface biotinylated and the CD44 immunoprecipitates immunoblotted with HRP-conjugated streptavidin. The positions of prestained $M_r$ markers are indicated on the left in kilodaltons.
known as β-elimination which removes oligosaccharides linked to serines and threonines by a chemical reaction. While all sulfate label was removed by this process, this treatment also destroyed the protein backbone as shown in the western blot of biotinylated CD44 protein (Fig. 5.13). Thus, it is apparent that TNFα does not stimulate the incorporation of [35S]sulfate into N-linked sugars on CD44, once [35S]sulfate amounts are normalized to CD44 protein amounts (Fig. 5.13). However, the data are not clear with respect to incorporation of [35S]sulfate into O-linked oligosaccharides on CD44. The methods used so far have resulted in inconclusive data. Experiments are ongoing to determine if CD44 is sulfated on O-linked sugars by size exclusion chromatography after β-elimination of the protein.

Proteins can also become sulfated post-translationally on tyrosine residues (reviewed in 218). CD44 immunoprecipitates were treated with aryl sulfatase from *Aerobacter aerogenes* to remove sulfate moieties from tyrosines. After TNFα stimulation, no sulfate label was removed after aryl sulfatase treatment, suggesting that CD44 was not sulfated on tyrosines (Fig. 5.14). An alternative method to examine sulfo-tyrosine on CD44 was used where [35S]sulfate labeled CD44 immunoprecipitates from TNFα stimulated SR91 cells were subjected to alkaline hydrolysis with barium hydroxide and examined by 2D-TLE. Alkaline hydrolysis was chosen as opposed to acid hydrolysis, for the analysis due to the extreme lability of the O-sulfate ester linkage in acid (218). Comparison of the hydrolyzed CD44 sample with sulfo-tyrosine standards stained with ninhydrin revealed the absence of radiolabel co-migrating with the standard (Fig. 5.15). As expected, no radiolabel from CD44 from unstimulated SR91 cells were observed to co-migrate with the sulfo-tyrosine standard. Thus CD44 does not incorporate [35S]sulfate on tyrosine residues in response to TNFα as shown by enzymatic and chemical removal methods. Pronase digestion of [35S]sulfate labeled CD44 immunoprecipitates resulted in a smear of radiolabel when run on a 1D-TLE, while in comparison, no radiolabel co-migrated with sulfo-tyrosine standards when a sample treated with barium hydroxide was run (Fig. 5.16). These data are consistent with CD44 expressing sulfated oligosaccharides or GAGs upon TNFα stimulation since pronase digestion of [35S]sulfate labeled CD44 would generate CD44 peptides with their branching sulfated side chains intact. Given that
Figure 5.14. Removal of [35S]sulfate on CD44 by sulfatase digestion. CD44 was immunoprecipitated from $5 \times 10^6$ [35S]sulfate labeled unstimulated or TNFα stimulated SR91 cells. Immunoprecipitates were subjected to digestion with aryl sulfatase from Aerobacter aerogenes (AS) or left undigested (-). Control immunoprecipitates in the absence of the immunoprecipitating mAb IM7.8.1 are indicated in lanes C. The positions of prestained $M_r$ markers are indicated on the left in kilodaltons.
Figure 5.15. Two dimensional sulfo-tyrosine analysis of CD44 from SR91 cells. CD44 was immunoprecipitated from 3 X 10^8 [35S]sulfate labeled unstimulated or TNFα stimulated SR91 cells. Immunoprecipitates were subjected to alkaline hydrolysis with barium hydroxide and the sample was electrophoresed on a TLC plate in two dimensions (see Materials and Methods for details). Tyrosine sulfate (Tyr-SO_4) and phenol red (PR) standards are as indicated and were visualized by ninhydrin staining. The origins are indicated by the circles in the bottom right hand corners.
Figure 5.16. One dimensional TLE analysis of CD44 from SR91 cells. CD44 was immunoprecipitated 5 X 10^7[^35]Sulfate labeled SR91 cells stimulated with TNFα for 24 hours. Immunoprecipitates were hydrolyzed in barium hydroxide (BaOH₂) or digested with pronase and run on a TLC plate in one dimension (see Materials and Methods for details). Tyrosine sulfate (Tyr-SO₄) and phenol red (PR) standards are indicated by the large filled circles and were visualized by ninhydrin staining. The origins are indicated by the circles at the bottom.
previous experiments show that CD44 is not inducibly sulfated on GAGs or N-linked sugars (Fig. 5.11 and Fig. 5.13), it is likely that the target of TNFα induced sulfation on CD44 is O-linked carbohydrate. However, at this point there is no direct evidence to confirm that the majority of $[^{35}S]$sulfate incorporation occurs on O-linked oligosaccharide moieties.

5.8 Induction of HA binding by pro-inflammatory cytokines and chemokines in peripheral blood mononuclear cells (PBMCs)

The SR91 cells have proven to be a very good model cell line to determine the mechanism by which TNFα could induce sulfation dependent HA binding and to further characterize the sulfated moiety on CD44. However, two caveats remain which prevent extrapolation of data from these cells to monocyte behaviour during an inflammatory response. First, SR91 cells were derived from a patient with acute lymphocytic leukemia, expressing surface markers characteristic of myeloid progenitor cells, not circulating monocytes. Combined with results showing the inability of SR91 cells to respond to a number of other pro-inflammatory factors, attention was focused on using freshly isolated peripheral blood for further experiments. Peripheral blood mononuclear cells (PBMCs) were isolated on Ficoll-hypaque gradients and treated with 10 ng/ml of TNFα or IL-1β for 24, 48, and 72 hours (data not shown). CD44 expression and HA binding ability of the PBMCs was determined by flow cytometry. While CD44 expression remained constant over the stimulation period, HA binding was induced optimally at 72 hours by TNFα in a subpopulation of PBMCs (Fig. 5.17). To determine whether the HA binding population consisted of monocytes, cells were selected on the basis of their larger size (forward scatter; FSC) and increased granularity (side scatter; SSC) as well as on the basis of CD14 expression, on the flow cytometer. Right-shifted HA binding histograms of unstimulated, size-selected PBMCs likely reflects the autofluorescence of dead cells which are present in the gated population. Double staining of the PBMCs with anti-CD14-PE and FL-HA revealed that the CD14 positive PBMCs contained a high FL-HA binding subpopulation of cells (Fig. 5.17). In contrast, no induction of HA binding was observed in PBMCs stimulated for up to 72 hours with IL-1β, again indicating the
Figure 5.17. Effect of pro-inflammatory cytokines and chemokines on HA binding ability on peripheral blood mononuclear cells. The expression of CD44 (A) and HA binding ability (B) on untreated cells, or PBMCs treated with TNFα, IL-1β, MIP-1α, and MCP-1 was determined by flow cytometry. Expression levels of CD44 and HA binding ability were determined using the anti-CD44 mAb IM7.8.1 and 5 µg/ml of FL-HA respectively. CD14 expression was determined using anti-CD14-PE. Size selected PBMCs were gated on the basis of their large size (forward scatter; FSC) and granularity (side scatter; SSC) on the flow cytometer. CD14 positive PBMCs were selected by virtue of gating on the CD14 positive population of cells double labeled with anti-CD14-PE and FL-HA. Unlabeled PBMCs were the negative control.
A  CD44 expression

B  FL-HA binding

PBMCS

Negative control
Unstimulated
TNFα
IL-1
MIP-1α
MCP-1

Size selected PBMCS

Unstimulated
TNFα
IL-1
MIP-1α
MCP-1

CD14+ PBMCS
selectivity of the TNFα response on CD44 in a subpopulation of PBMCs similar to the case with SR91 cells.

Treatment of freshly isolated PBMCs with 50 ng/ml of MIP-1α or MCP-1 for 72 hours led to a slight increase in CD44 expression but more importantly these pro-inflammatory chemokines were able to induce FL-HA binding in a size selected population of PBMCs (Fig. 5.17). Since these chemokines are involved in activating adhesive function in monocytes during an inflammatory response, the monocyte population was examined for its ability to bind FL-HA. Size selected PBMCs could be induced to bind HA and using CD14 as a marker for monocytes, it was observed that this subpopulation of PBMCs included cells which could be induced to bind HA upon stimulation with MIP-1α or MCP-1 (Fig. 5.17). This result is in marked contrast to the effects of MIP-1α or MCP-1 on the HA binding ability of SR91 cells (Fig. 5.4). Thus, it appears that the pro-inflammatory cytokine TNFα and the monocyte-specific inflammatory chemokines MIP-1α and MCP-1 can convert CD44 from an inactive form that cannot bind HA to an active, HA binding molecule on a subpopulation of PBMCs that include CD14 positive monocytes.

5.9 TNFα induction of HA binding in PBMCs is sulfation dependent

To determine whether sulfation was required for TNFα-induced HA binding in a subpopulation of PBMCs, freshly isolated PBMCs were incubated with 50 mM sodium chlorate during the TNFα stimulation period. Chlorate inhibits ATP sulfurylase which generates adenosine 3’ phosphate 5’ phosphosulfate (PAPS) from ATP and sulfate. PAPS is the sulfate donor in sulfate transfer reactions. Using two separate donors, it was observed that TNFα was able to induce FL-HA binding in a size selected population of PBMCs (Fig. 5.18B). These size selected cells were chosen on the basis of their large size and granularity. Induction of HA binding was almost completely blocked by incubation with 50 mM sodium chlorate, indicating that HA binding was sulfation dependent (Fig. 5.18B). Levels of CD44 expression were high but fairly heterogeneous on the PBMCs from both donors, therefore it was not readily apparent that TNFα caused an increase in surface CD44 expression (Fig. 5.18A).
Figure 5.18. Effect of chlorate on TNFα-inducible HA binding on PBMCs. The expression of CD44 (A) and HA binding ability (B) was determined on PMBCs from two different donors by flow cytometry. PBMCs were left unstimulated or treated with TNFα or TNFα and 50 mM sodium chlorate for 72 hours. Expression levels of CD44 were determined using the anti-CD44 mAb IM7.8.1. CD44 expression on total PBMCs are shown in (A) and FL-HA binding profiles of size selected PMBCs are shown in (B). Unlabeled PBMCs are the negative control.
To determine if sulfation dependent HA binding was induced by TNFα in monocytes, the CD14 positive population of PBMCs was examined by double labeling with anti-CD14-PE and FL-HA. In human peripheral blood, CD14 is considered the classical marker for monocytes, although the integrin Mac-1 could also have been used to select for the monocyte population. TNFα could induce HA binding in this population of cells from two independent blood donors (Fig. 5.19B). Additionally, this binding may be partially dependent on sulfation since incubation with 50 mM sodium chlorate during the stimulation period reduced HA binding significantly (Fig. 5.19B), although some variability in this effect was observed between different donors. Expression of CD14 increased on PBMCs upon TNFα stimulation (Fig. 5.19A) indicating that TNFα may upregulate the expression of CD14 on monocytes and may even cause the differentiation of promyelomonocytic cells into monocytes, thus increasing the number of CD14 positive monocytes. Chlorate treatment of the cells did not affect CD44 or CD14 expression and did not decrease cell viability as assessed by trypan blue exclusion.

To examine whether TNFα induced HA binding by monocytes specifically, PBMCs were purified by adherence to plastic to isolate the monocyte/macrophage population. Both unpurified PBMCs and purified monocytes expressed similar levels of CD44 on the cell surface (Fig. 5.20A). Investigation of the HA binding properties of these cells demonstrated that TNFα induced binding in sized selected PBMCs and purified monocytes (Fig. 5.20B). HA binding appeared to be dependent on sulfation in purified monocytes as determined by chlorate treatment to block sulfation (Fig. 5.20B). Chlorate did not have as significant an effect on TNFα-inducible HA binding by sized selected PBMCs from this particular donor. Using an anti-CD14-PE mAb, a more significant CD14 positive subpopulation of cells was observed from monocytes purified on plastic compared to sized selected PBMCs, as expected (Fig. 5.21A). Treatment of purified monocytes with TNFα caused an upregulation in CD14 expression as determined by flow cytometry. This effect was not as dramatic prior to purification of monocytes by adherence to plastic (Fig. 5.21A). Double labeling of PBMCs and purified monocytes with anti-CD14-PE and FL-HA revealed that TNFα could activate HA binding and that this binding was reduced by 50 mM sodium chlorate in
Figure 5.19. Effect of chlorate on TNFα-inducible HA binding on CD14 positive PBMCs. The expression of CD14 (A) and HA binding ability (B) was determined on PBMCs from two different donors by flow cytometry. PBMCs were left unstimulated or treated with TNFα or TNFα and 50 mM sodium chlorate for 72 hours. Expression levels of CD14 were determined using anti-CD14-PE. Surface CD14 levels on total PBMCs are shown in (A) and the FL-HA binding profiles of CD14 positive PMBCs are shown in (B). Unlabeled PBMCs are the negative control.
Figure 5.20. Comparison of the effect of chlorate on TNFα-inducible HA binding on PBMCs and purified monocytes. The expression of CD44 (A) and HA binding ability (B) was determined on PMBCs and monocytes purified by adherence on plastic by flow cytometry. PBMCs or purified monocytes were left unstimulated or treated with TNFα or TNFα and 50 mM sodium chlorate for 72 hours. Expression levels of CD44 were determined using the anti-CD44 mAb 3G12. Surface CD44 levels on total PBMCs and total purified monocytes are shown in (A) and the FL-HA binding profiles of size selected PMBCs and size selected purified monocytes are shown in (B). Unlabeled PBMCs or unlabeled purified monocytes are the negative control.
A  CD44 expression

PBMCs  Purified monocytes

Cell number

Fluorescence intensity

Negative control  Unstimulated  TNFα  TNFα/50 mM chlorate

B  FL-HA binding

Size selected PBMCs  Size selected purified monocytes

Cell number

Fluorescence intensity

Unstimulated  TNFα  TNFα/50 mM chlorate
Figure 5.21. Comparison of the effect of chlorate on TNFα-inducible HA binding on the CD14 positive subpopulation of PBMCs and purified monocytes. The expression of CD14 (A) and HA binding ability (B) was determined on PMBCs and monocytes purified by adherence to plastic by flow cytometry. PBMCs and purified monocytes were left unstimulated or treated with TNFα or TNFα and 50 mM sodium chlorate for 72 hours. Expression levels of CD14 were determined using anti-CD14-PE. CD14 expression on total PBMCs and purified monocytes are shown in (A) and the FL-HA binding profiles of CD14 positive PMBCs and purified monocytes are presented in (B). Unlabeled PBMCs and unlabeled purified monocytes are the negative control.
A  CD14 expression

Size selected PBMCs  Size selected purified monocytes

Cell number

Fluorescence intensity

10^1  10^2  10^3  10^4

Unstimulated

TNFα

TNFα/50 mM chlorate

B  FL-HA binding

CD14+ PBMCs  CD14+ purified monocytes

Cell number

Fluorescence intensity

10^1  10^2  10^3  10^4
PBMCs when gating upon the CD14 population. However, the effect of blocking sulfation with chlorate was more readily observed when the CD14 positive purified monocytes were examined (Fig. 5.21B). It is evident that patient variation may account for differences in observed TNFα inducible HA binding and the effect of chlorate on this binding, and more samples will be needed to state unequivocally that sulfation is the mechanism by which TNFα induces HA binding in monocytes. However, the population of cells affected by TNFα treatment are most likely CD14 positive monocytes and, similar to the SR91 cells, conversion of peripheral blood monocytes from a non-HA binding phenotype to an HA binding population may also be mediated by sulfation.

5.10 Can other cell lines bind HA in a sulfation dependent manner?

Given that sulfation is required for TNFα-inducible HA binding by SR91 cells and may be involved in TNFα-inducible HA binding by peripheral blood monocytes, other cell types were examined to ascertain whether sulfation is a general mechanism to regulated HA binding in myeloid cells. Generally, primitive myeloid cells express high levels of CD44 in the bone marrow and bone marrow stromal cells produce abundant amounts of HA. Thus it was relevant to investigate whether a homogeneous population of primitive myeloid cells could bind HA in a sulfation dependent manner. To that end, the KG1a cell line was used because its differentiation is blocked at an early stage of myelopoiesis; expression of CD34 implies that these cells are early progenitors in the myeloid lineage (ATCC). KG1a cells express high levels of surface CD44 and are able to bind HA constitutively (Fig. 5.22). This HA binding is CD44-mediated as reported elsewhere (219). More significantly, treatment of KG1a cells for 24 hours with increasing amounts of the PAPS synthesis inhibitor, sodium chlorate, resulted in a dose-dependent decrease in HA binding, with almost complete inhibition at 200 mM sodium chlorate (Fig. 5.22). For all doses of chlorate, CD44 expression was high, and did not vary significantly (Fig. 5.22). No effect on cell viability was observed as determined by trypan blue exclusion except at the highest dose of chlorate. Nonetheless, CD44-mediated HA binding is dependent on sulfation in an early myeloid progenitor cell line. Taken together, these studies indicate that sulfation may be an important regulator of
Figure. 5.22. Effect of chlorate on HA binding ability of the KG1a cell line.
CD44 expression levels and HA binding ability was determined by flow cytometry on the KG1a myeloid progenitor cell line. Untreated KG1a cells or cells treated with 50, 100, 150, and 200 mM sodium chlorate for 24 hours and assessed for their ability to bind to 5 μg/ml of FL-HA. CD44 expression was determined using the anti-CD44 mAb IM7.8.1. Unlabeled KG1a cells were the negative control.
CD44-mediated adhesion in monocytes and cells of the myeloid lineage. In addition, induction of sulfation dependent HA binding by TNFα suggests a function for CD44 mediated adhesion at inflammatory sites.
6.0 Triton X-100 insolubility of CD44 in adherent cell lines

A significant proportion of CD44 (30-35%) is found to be insoluble after Triton X-100 extraction of NIH 3T3 cells (Fig. 3.1A). This is in agreement with the observed Triton X-100 insolubility of CD44 in fibroblasts and some epithelial cells (60, 85, 119, 128, 145, 147, 220). CD44 insolubility has been attributed to an association with the detergent insoluble cytoskeletal elements of the cell and has been described for other molecules that bind to extracellular matrix components (221-225). By contrast, CD44 is relatively Triton X-100 soluble in lymphocytes and this may reflect the less organized cytoskeleton found in non-adherent cells such as leukocytes (122, 147). If CD44 insolubility in Triton X-100 can be attributed to an association between CD44 and the cytoskeleton, a number of mechanisms have been postulated to explain the interaction. CD44 has been shown to interact either directly or indirectly with actin (119, 128, 145) and intermediate filaments (85). It has been demonstrated that the cytoskeletal linker molecule ankyrin can interact \textit{in vitro} with CD44 (58, 120, 226, 227). Another family of proteins that are thought to regulate actin-plasma membrane interactions, the ERM (ezrin, radixin, moesin) family (reviewed in 228, 229, 230) were shown to interact, both \textit{in vitro} and \textit{in vivo}, with CD44 (151-153, 231). However, contradictory data has been reported regarding the association between CD44 and the actin cytoskeleton. Perschl \textit{et al.} were unable to detect a decrease in the amount of Triton X-100 insoluble CD44 or actin as a result of DNase I treatment of fibroblasts, immunofluorescent staining of CD44 was not affected by disruption of the actin cytoskeleton with cytochalasin D or cytochalasin B (147, 155, 205), and nocodazole disruption of microtubules also had no effect on CD44 solubility (147). Additionally, CD44 did not co-localize with phalloidin stained actin stress fibres in fibroblasts (85, 147, 154, 205).
6.1 CD44 migrates with the low density lipid fraction

Together with the role of CD44 in cell motility and evidence that the cytoplasmic domain of CD44 could interact with cytoskeletal proteins, Triton X-100 insolubility was interpreted to signify that CD44 was associated with the actin cytoskeleton. However, deletion of the CD44 cytoplasmic domain did not reduce the insolubility of the mutant upon detergent extraction, indicating that associations observed between the cytoplasmic domain of CD44 and components of the actin cytoskeleton may not be responsible for the insolubility of CD44 in Triton X-100 (60, 122, 147, 155, 220). Protein lipid interactions in the cell membrane however, could account for the observed insolubility of CD44 in Triton X-100. A number of cellular components that are considered detergent insoluble have been localized to detergent insoluble lipid domains, including glycosphingolipids (159), GPI-anchored proteins, and membrane associated signaling molecules (171, 172).

Flotation of Triton X-100 lysed NIH 3T3 cells on sucrose revealed that a proportion of CD44 migrated with fractions containing low density material (Fig. 3.2A). After these experiments were performed, others reported similar findings (155, 232, 233). Migration of a significant amount of p60yes protein from NIH 3T3 cells and the presence of a cloudy band of protein and lipid at the interface of 5% and 30% sucrose were consistent with published accounts and led to the belief that fractions 2-4 represented low density material. However, since the dye front of the SDS-PAGE gels were run off, the membrane could not be probed with biotinylated cholera toxin B subunit to confirm the presence of G\textsubscript{M1} ganglioside in fractions 2-4 of the gradients. G\textsubscript{M1} ganglioside, a major component of low density lipid fractions, is excluded from detergent soluble material (159) and is a target for cholera toxin B binding (234). A small amount of CD44 was found in the insoluble pellet at the bottom of the gradient as observed by others (155). This could be due to a combination of insufficient washing of the pellet and non-specific trapping of CD44 in the pellet during centrifugation, since CD44 was not observed in the pellet of the gradients from NIH 3T3 cells transfected with the CD44 cytoplasmic deletion mutant or from
CD44 in L cells. Alternatively, CD44 in the pellet at the bottom of the gradient could be attributed to the presence of cytoskeletally associated CD44 in NIH 3T3 cells.

A small amount of CD44 migrated in the low density fractions of sucrose gradients from NIH 3T3 cells lysed in 60 mM n-octyl-β-D-glucopyranoside (octyl glucoside) (Fig. 3.2B). While octyl glucoside was not able to completely inhibit the migration of CD44 to the low density domains, it was able to solubilize what potentially was cytoskeletally associated CD44 since CD44 was no longer found in the pellet fraction. It is possible that the use of higher concentrations of octyl glucoside could disperse lipid associations more effectively, thus preventing the localization of CD44 to the low density lipid fraction completely. Alternatively, certain associations may be refractory to the action of octyl glucoside and its ability to dissociate lipid-protein interactions. In human neutrophils, octyl glucoside was used to separate cytoskeletal structures from caveolae, a type of detergent insoluble low density lipid domain. However, it was reported that the caveolae marker protein, caveolin, as well as α-actinin, p58cfgr, and p53/56lyn remained insoluble in octyl glucoside (235). This group speculated that the octyl glucoside insolubility reflected a very tight association between lipid domains and a fraction of highly polymerized cytoskeleton. It may be that octyl glucoside is not efficient at dispersing low density lipid associated CD44, not because of the concentration of octyl glucoside used, but because a proportion of CD44 in low density fractions forms a tight complex with other CD44 molecules via its transmembrane domain together with cytoskeletal elements via its cytoplasmic domain. If this were true, it could be hypothesized that CD44 lacking a cytoplasmic domain would not migrate in the low density fractions in octyl glucoside since direct interaction between the cytoplasmic tail of CD44 and the cytoskeleton could not occur. This could not be assessed experimentally since the CD44 cytoplasmic deletion mutant was transfected into NIH 3T3 cells which endogenously express a full length CD44 molecule and transmembrane interactions would allow the complexes to form.

The presence of large protein-lipid complexes refractory to octyl glucoside solubilization may be further explained by the following. Low density fraction associated CD44 could be comprised of several types of lipid fractions of varying size, containing different complements of...
proteins. Smaller vesicles could be represented by the pool of CD44 that migrates to the low
density fraction in Triton X-100 but not in octyl glucoside, while larger complexes would migrate
in the low density fraction in both detergents and perhaps also be trapped in the pellet fraction of
the Triton X-100 gradients. In support of this theory, CD44 from peripheral blood lymphocytes
has been reported to migrate to the low density fractions of sucrose gradients run in octyl glucoside
(232), although in this case more CD44 migrated to the low density fraction after solubilization in
octyl glucoside compared to 0.5% Triton X-100. Additionally, it was observed that centrifugation
of Triton X-100 soluble lysates from NIH 3T3 cells on sucrose gradients led to the localization of
CD44 in the low density fraction, although the amounts were significantly reduced compared to
Fig. 3.2 (data not shown). Perschl et al. reported that centrifugation of the Triton X-100 soluble
material on sucrose gradients resulted in the bulk of CD44 (approximately 80%) in the soluble
fractions. While the whereabouts of the remaining 20% of CD44 was not discussed, careful
examination of the data revealed the presence of CD44 bands in the low density fractions (155).

6.2 The transmembrane domain of CD44 mediates its migration with the low
density lipid fraction

With the observation that approximately 20% of CD44 expressed in NIH 3T3 cells could
be found associated with the low density fractions of sucrose gradients, it became apparent that as
was the case with the Triton X-100 solubilization experiments, the cytoplasmic domain of CD44
did not cause the migration of CD44 to low density fractions (Fig.3.4). At about this time, it was
shown that the transmembrane domain of CD44 was responsible for its detergent insolubility.
Chimeric CD44 molecules containing the transmembrane domains of CD45 or the CD3ζ chain
were completely solubilized in Triton X-100 (55). Thus, it was theorized that both the full length
CD44 molecule in NIH 3T3 cells and the cytoplasmic domain deletion mutant of CD44 could
associate with low density material on sucrose gradients due to the presence of an intact
transmembrane domain. Here, it was shown that the CD44 chimeric molecule containing the
CD45 transmembrane domain centrifuged on sucrose did not migrate in the low density fractions
(Fig. 3.5), confirming that the transmembrane domain of CD44 is responsible for migration with low density material.

The CD44 transmembrane domain can mediate the self-association of CD44 on the cell surface of T cell lines to promote HA binding and CD44 dimers were observed in NIH 3T3 cells (53). In that study, a chimeric molecule containing the CD4 extracellular domain and the CD44 transmembrane and cytoplasmic domains was transfected into T cell lines and NIH 3T3 cells expressing endogenous CD44. Associations between endogenously expressed CD44 and the chimeric molecules were observed. Thus, in NIH 3T3 cells transfected with the CD44 cytoplasmic deletion mutant, the tailless CD44 molecule may self-associate or form heterodimers with the full length molecule and this may mediate the migration of tailless CD44 to low density lipid fractions. Transmembrane domain mediated associations have been documented; CD45 associates with CD45AP via their respective transmembrane domains (236) and glycophorin A, another cell surface molecule, also forms homodimers. Dimerization of glycophorin A is mediated by a noncovalent interaction between the transmembrane regions, indicating that specific associations can occur between identical α-helices (237, 238). To extend my findings, it would be useful to show that the chimeric CD4/CD44 molecules form noncovalent associations with endogenous CD44 in the low density fractions but not in the soluble fractions of sucrose gradients. Expression of the non-HA binding CD4/CD44 chimera in T cells reduced HA binding by possibly competing for, and disrupting, endogenous CD44-CD44 interactions, suggesting that HA binding is enhanced upon CD44 self-association. It is therefore intriguing to speculate that dimerization of CD44 in low density lipid domains potentiates HA binding.

CD44 can incorporate $[^3]$Hpalmitic acid and acylation is thought to be one mode by which some proteins are localized to low density lipid fractions. CD44 has been shown to be palmitoylated in murine and human T cell lines although it is not known whether this occurs on a cysteine in the transmembrane domain or the cytoplasmic domain (226, 239). However, incorporation of $[^3]$Hpalmitic acid on CD44 in the same cells was never observed in my experiments (data not shown). It is even less likely that the palmitoylation of CD44 mediates its...
migration with the low density fractions in light of recent findings of Schieffele et al. This group used influenza virus haemagglutinin as a model protein to analyze the interaction of type I transmembrane proteins with sphingolipid-cholesterol rich microdomains. They found that lipid raft association is an intrinsic property encoded in the protein sequence of the transmembrane domain. Mutant haemagglutinin molecules with a foreign transmembrane domain lost their ability to associate with lipid microdomains despite being palmitoylated (240). Further mutations of the haemagglutinin transmembrane domain revealed a requirement for hydrophobic residues for contact with the exoplasmic leaflet of the membrane. Indeed, the exoplasmic side of the CD44 transmembrane domain is quite hydrophobic, consisting mainly of isoleucine and leucine residues. It may be that these hydrophobic residues mediate protein-lipid interactions between transmembrane proteins and cholesterol. It should be noted however, that not all transmembrane proteins localize to low density lipid fractions. The transferrin receptor, CD45, and $\beta_1$ integrins are excluded from these fractions (167, 241).

6.3 Functional significance of CD44 localization in low density lipid fractions

The biological significance of CD44 localization to low density lipid fractions is not clear but there are hints as to its function. Several reports indicate that aggregation of CD44 molecules on the cell surface can facilitate HA binding. Localization in low density lipid fractions may increase local concentrations of CD44, mediated by aggregation or dimerization via the transmembrane domain, thus facilitating HA binding. For example, induction of HA binding by the anti-CD44 mAb IRAWB 14 requires the maintenance of the divalent nature of the antibody and the authors conclude that IRAWB 14 induces HA binding by directly influencing the distribution of CD44 on the cell surface (123), potentially by aggregating CD44 molecules. Substitution of the CD44 transmembrane domain with that of CD3ζ, which forms disulfide-linked homodimers, resulted in the upregulation of HA binding in the AKR T lymphoma cell line (55). Treatment of CD44 transfected Jurkat T cells with PMA induced the disulfide-bonded dimerization of CD44 and HA binding (54, 242). The generation of novel CD4/CD44 chimeras and CD44-negative
fibroblasts from CD44 knockout mice in the intervening time since the experiments detailed here were completed, should allow a better assessment of the dimerization status of CD44 in low density lipid fractions. Similarly, with the availability of reagents such as biotinylated HA, it should now be possible to determine whether CD44 found in low density sucrose fractions is able to bind HA. However, HA binding by low density fraction associated CD44 may have to be assessed in lymphoid cells since NIH 3T3 cells do not bind HA constitutively and it is not known whether CD44 migrating to low density fractions is able to bind HA on NIH 3T3 cells.

Considering that the anti-CD44 mAb, IRAWB 14 is able to induce HA binding in NIH 3T3 cells, it would be beneficial to investigate whether IRAWB 14 can increase the proportion of CD44 found in the low density fraction by virtue of CD44 crosslinking, and whether this fraction binds biotinylated HA by western blot.

Detergent insoluble lipid rafts have been proposed to function as platforms for signal transduction and membrane trafficking (157) and are enriched with a number of signal transducing molecules, GPI-linked proteins, actin, and actin-binding proteins (172, 179, 243). Recently, low density lipid domains have been identified as sites where receptor-induced signaling and cytoskeletal reorganization integrate, as demonstrated for T cell activation and signaling through the high affinity receptor for IgE, the FceRI receptor (180, 182-186). In NIH 3T3 cells, reactivity to an anti-c-src antibody was largely localized to the low density lipid fractions. Since the antibody used in these experiments crossreacts to both p60<sup>src</sup> and p62<sup>yes</sup> (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), it is difficult to state with any certainty that the reactivity in the low density fractions is due to either p60<sup>src</sup> (src) or p62<sup>yes</sup> (yes). Fibroblasts express both src and yes and it has been demonstrated that in MDCK cells, yes migrates almost exclusively to the low density fractions on sucrose gradients (171, 172). The localization of src family kinases to low density fractions is thought to be as a consequence of dual acylation of the N-terminal of these proteins (204, 244, 245). In fact, it is thought that src kinases containing consensus sequences for both myristoylation and palmitoylation migrate to lipid rafts while the presence of a myristate anchor alone, as is the case for src, is insufficient to mediate migration with low density lipid fractions.
However, there is evidence that src can migrate to the low density fractions on sucrose gradients despite the absence of palmitoylation (171, 172). From my experiments, it is not clear whether the c-src antibody reactive protein in the low density fractions is src, yes, or a combination of both proteins; immunoblotting the fractions with src-specific and yes-specific antibodies would resolve this issue. In addition, the presence of an 80 kD band on the anti-c-src western blots was confounding but probably represented non-specific protein reactivity which was observed by others in the laboratory with lysates from a number of different cell types (data not shown). In any case, the majority of anti-c-src reactive protein migrated to the higher fractions of the sucrose gradient in agreement with published data (171, 172) suggesting that the experimental procedure was successful in isolating low density lipid fractions and that there may be a correlation between low density fractions observed in these experiments and the lipid domains or lipid rafts reported by others.

Probing fractions from NIH 3T3 sucrose gradients with an anti-actin mAb revealed that most of the anti-actin reactive protein was found at the bottom of the gradient, consistent with actin being a major component of the cytoskeleton. Actin found in the pellet and soluble fractions probably represents polymerized and monomeric actin, respectively. The migration of an anti-actin reactive protein in the low density fraction is likely to reflect the presence of globular actin (G-actin) in these fractions. It has been reported that G-actin from lung endothelium can migrate to low density lipid rafts, as determined by microsequence analysis and confirmed by binding to Gc-globulin (172). The presence of G-actin in low density fractions from NIH 3T3 can therefore be confirmed by binding to Gc-globulin, since it is specific for G-actin (246). Another cytoskeletal protein observed in the low density fractions from NIH 3T3 cells was annexin II. This protein has been reported to migrate with low density lipid rafts in MDCK cells and lung endothelium (171, 172, 207). In NIH 3T3 cells, approximately 10% of total annexin II migrated to the low density fractions. However, it has been reported that tetrameric complexes consisting of annexin II dimers and dimers of its associated protein, p11, are major components of the Triton X-100 low density fraction from MDCK cells in the presence of calcium (207). The sucrose gradients detailed in this
thesis were prepared in the absence of calcium and in the presence of a calcium chelator, EDTA, which may explain the discrepancies between the amount of annexin II observed in the low density fractions described here compared to MDCK cells. Despite the different solubilization conditions, the presence of annexin II in the NIH 3T3 low density fractions further supports the correlation between these fractions and lipid rafts. The presence of annexin II in low density lipid rafts is intriguing by virtue of its formation of a tetrameric complex with p11, its abundance in lipid rafts, and the fact that it is a cytoskeletal and membrane phospholipid interacting protein (reviewed in 208). In fact, in mammary epithelial cells, CD44 and annexin II partition to the same type of lipid rafts as demonstrated by experiments in living cells (233). Crosslinking of CD44 with anti-CD44 mAbs recruited annexin II to the cytoplasmic leaflet of the CD44 clusters. Furthermore, redirection of actin bundles to these clusters was observed and these CD44/annexin II lipid raft complexes were stabilized by the addition of GTPγS or phalloidin. Thus it appears that in these cells, the majority of CD44 interacts with annexin II in lipid microdomains in a cholesterol-dependent manner and that the domains interact with the underlying cytoskeleton.

If one considers the migration of CD44 with low density lipid fractions as a means to assemble a CD44 signaling complex, it is useful to draw analogies to FceRI. Aggregation of FceRI is required for its association with detergent resistant membrane rafts (181-183), and this is thought to be mediated by dimerization via the transmembrane domains of the α and/or γ subunits of FceRI (184). Thus, it is tempting to speculate that CD44 also dimerizes in the low density lipid fraction (as discussed above) in order to initiate formation of a signaling complex. In view of the fact that other transmembrane proteins form signaling complexes via dimerization or oligomerization, the TCR among these, an alignment was performed of the transmembrane domains of proteins involved in signal transduction in a variety of immune processes (247). It became apparent upon analysis of these transmembrane domains, that many of these proteins contained a cysteine in the last 3-6 amino acids of the transmembrane domain. Since cysteines appear to be critical for localization of src family kinases to low density fractions, the importance of this cysteine for CD44 migration to the low density fraction was assessed. Cysteine 268 of the
CD44.1 allele was mutated to an alanine and transfected into NIH 3T3 cells endogenously expressing the CD44.2 allele. It was determined that cysteine 268 in the transmembrane domain of CD44 was not required for migration to low density fractions since the mutated molecule was localized there (data not shown). Mutation of the transmembrane cysteine 268 to a serine did not abolish the noncovalent association between the CD4/CD44 chimera to endogenously expressed CD44 (53), indicating that this cysteine is not critical for self-association of CD44. Recently, a report suggested that the constitutive association between CD44 and p56\textsuperscript{ick} (Ick) in T lymphocytes, and CD44 and p59\textsuperscript{lyn} (Lyn) in B lymphocytes, was mediated by the transmembrane cysteine of CD44 (247). In addition, Ick and Lyn were shown to be translocated with CD44 to the Triton X-100 resistant cell fraction. Mutation of the transmembrane cysteine of CD44 completely abolished the interaction with Ick and Lyn, while truncation of three tandem arginines at the beginning of the CD44 cytoplasmic domain significantly decreased it.

A theme is emerging with respect to low density lipid rafts as specialized signaling domains on the cell surface membranes of a number of cell types, although haematopoietic cells appear to be the most well-characterized. Potentially, transmembrane molecules assemble with low density lipids during the protein sorting process in the Golgi apparatus by virtue of aggregation via their transmembrane domains (175). Signal transducing molecules, such as the src family kinases may translocate to the low density lipid fraction upon dual acylation of its N-terminal region. Another view is that since src cannot be palmitoylated, that it is the unpalmitoylated cysteine in the N-terminal of src kinases which determines migration to lipid rafts (204). If this is the case, it can be envisaged that src family kinases interact with transmembrane proteins in low density lipid fractions via their respective cysteines; the cysteine in the N-terminal of the src kinases and the transmembrane cysteine of integral membrane proteins. Supporting evidence for a cysteine-cysteine non-covalent interaction comes from work in T lymphocytes characterizing the association between CD4 and Ick (248-250) coordinated by a Zn\textsuperscript{2+} ion, not disulfide bonding (251).

Although CD44 localization to low density fractions may facilitate formation of a CD44 signaling complex, it is unclear what the consequences of CD44 signal transduction would be in...
NIH 3T3 cells. Functionally, CD44 may be localized to low density lipid fractions in NIH 3T3 cells to facilitate turnover of CD44 on the cell surface and internalization and degradation of HA. The mechanisms of CD44 turnover are not very well characterized, although it has been reported that CD44 is not internalized via clathrin-mediated coated pits (252) and that CD44 can be shed from the surface of transformed fibroblasts (253). It has become evident in the literature, that caveolae, a type of detergent insoluble lipid raft rich in caveolin protein, plays a role in transcytosis (177) and in potocytosis, the transport of small molecules of less than 1 kD into cells (reviewed in 254). An example of uptake of small molecules by potocytosis is that of folate where the GPI-anchored folate receptor is clustered on the cell surface in caveolae (255, 256). In an analogous fashion, the migration of CD44 to low density lipid fractions may be a means by which CD44 is internalized and may possibly correlate with localization in caveolae. In support of this, the surface staining pattern of CD44 in SV-3T3 transformed fibroblasts (253) resembled patches similar to those of the folate receptor on epithelial cells (255, 256). However, at this time, co-localization of the major protein component of caveolae, caveolin, with CD44 has not been observed in NIH 3T3 cells by immunofluorescence microscopy or by blotting of low density sucrose fractions. Thus, it cannot be concluded that CD44 localizes in caveolae to mediate its internalization.

Degradation of HA has important functional consequences. It allows cell migration through the extracellular matrix and basement membrane during tumour invasion, wound healing, and in embryonic development. HA degradation has been implicated in angiogenesis (257) and the vascularization of new tumours. It has been observed that abundant HA is present on epidermal keratinocytes (258) and that approximately 50% is associated with HA synthetase while the other 50% is bound to CD44. HA bound to CD44 on the cell surface may be targeted for degradation via a pathway that involves localization of CD44 in low density lipid fractions. In fact, when Culty et al. reported that CD44 was involved in HA degradation, they demonstrated that FL-HA was taken up in granules or patches (253) which are reminiscent of the folate receptor patches associated with caveolae (255, 256). HA was subsequently enzymatically degraded in acidified lysosomes by hyaluronidases. One inconsistency with this data is that fibroblasts in general, and
NIH 3T3 cells in particular, do not bind soluble FL-HA constitutively, as determined by flow cytometry. However, these data suggest that the requirements for binding and degrading HA in the context of a structurally ordered extracellular matrix may be less stringent than for binding of HA in solution.

6.4 Differential binding properties of L cell lines

The presence of the three different HA binding phenotypes of CD44 in the L cell lines correlated with differences in CS synthesis and oligosaccharide processing. In the presence of both CS synthesis and complete N- and O-linked oligosaccharide processing, CD44 exhibits an inducible HA binding phenotype in L cells. With the loss of CS synthesis, CD44 has a non-inducible, non-binding phenotype. When both CS synthesis and oligosaccharide processing are defective, inducible HA binding by CD44 is restored and some constitutive binding is observed (Fig. 4.1).

The cell lines used in this study are well characterized by a variety of assays, including anion exchange chromatography of labeled GAGs, and they have remained stable for over 5 years of observation (190, 191). Studies on these cell lines thus provide an advantage over enzymatic and inhibitor studies where it is often difficult to determine their precise effects. For example, treatment of these cells with chondroitin ABC lyase from different commercial sources had different effects (192). Likewise, treatment of the cell with the GAG inhibitor p-nitrophenyl β-D-xylopyranoside, produced different results to those obtained with chondroitin ABC lyase, and varied with the concentration used (192).

6.5 Effect of chondroitin sulfate and sulfation on IRAWB 14 inducible HA binding of CD44

Sulfation of CD44 has previously been reported and has been attributed to the presence of sulfated proteins or carbohydrates on CD44H, and to the addition of CS on the higher M, forms of CD44 (37). In my studies, both CS-modified and CD44H forms of CD44 were sulfated (Fig. 130).
4.4). However, the absence of the CS-modified CD44 form in sog9 cells resulted in the loss of IRAWB 14 inducible HA binding. Exogenously added CSA or CSC did not restore inducible FL-HA binding in sog9 cells, suggesting that CS had to be attached to a cell surface protein, likely CD44, for it to affect IRAWB 14-induced HA binding (192). The treatment of L cells, which contain CS-modified CD44, with sodium chlorate abolished IRAWB 14-inducible binding, whereas no inhibitory effect was observed in sog8 cells where CS synthesis does not occur (Fig. 4.5). This is consistent with the possibility that sodium chlorate inhibits HA binding in L cells by preventing the sulfation of chondroitin (259).

As demonstrated by gro2C cells, HS is not required for IRAWB 14-inducible HA binding by CD44 although it is required for infection by HSV-1. Transfection of sog9 cells with pools of HeLa cell cDNAs to restore HSV-1 infectivity resulted in the identification of EXT1, an ER-resident type II transmembrane glycoprotein responsible for altered expression of heparan sulfate (260). Mutations in the tumour suppressor gene EXT1 leads to hereditary multiple exotoses which is characterized by skeletal abnormalities in humans and the subsequent development of chondrosarcomas and osteosarcomas. However, transfection of sog9 cells with EXT1 did not restore IRAWB 14 inducible HA binding by CD44 (192), although these cells were able to synthesize heparan sulfate as determined by HPLC (260).

The results in this study appear to conflict with those reported by Lesley et al. (117) who found a negative regulatory role for both oligosaccharides and GAGs. Attempts to reconcile these differences by performing enzymatic and inhibitor studies generated data suggesting that these reagents have additional activities that led to non-specific effects (192). It is possible that differences could arise from the following observations: the parental L cell clones used in each case were different and had different HA binding capabilities, the effect of CS can depend on the glycosylation state of the cell; and growing cells in different glucose concentrations can change their glycosylation state and HA binding ability (113).
6.6 Characterization of the glycosylation defect present in sog8 cells and its effect on HA binding

The core protein of CD44H is predicted to be 37 kD (38-40), yet the first synthesized CD44 protein detected after $^{35}$S methionine and cysteine labeling had an apparent $M_r$ of 62 kD (Fig. 4.6). Pulse chase analysis and treatment of CD44 immunoprecipitates with PNGase F and O-glycosidase indicated a defect in both N- and O-linked processing in sog8 cells, but not a complete absence of glycosylation (192).

In sog8 cells, the absence of the majority of O-linked oligosaccharides and terminal sugar moieties on N-linked carbohydrates enhanced the HA binding ability of CD44. Further analysis of sog8 cells indicated the lack of terminal sialic residues and their relative resistance to ricin suggested decreased expression of galactose (Gal) residues, leaving carbohydrates expressing terminal N-acetyl glucosamines (GlcNac). Due to the acquisition of endoH resistant CD44 species, it is evident that CD44 on sog8 cells express at least one N-linked GlcNac. It is interesting to note that addition of the first N-linked GlcNac during N-linked oligosaccharide processing, enhanced HA binding by soluble CD44-Ig fusion proteins (115). However, treatment of L and sog9 cells with neuraminidase alone did not change their ability to bind FL-HA (data not shown), suggesting that sialic acids alone may be insufficient to impede HA binding or that enzymatic digestion did not go to completion. While an increase in binding of PNA lectin was observed (data not shown), we could not easily determine if all sialic acids had been removed. This result agrees with Lesley et al. (117), who also found that treatment of L cells with neuraminidase did not affect the HA binding ability of CD44. However, it is of note that neuraminidase treatment of soluble, purified CD44-Ig fusion protein did enhance its ability to bind HA (116) and that expression of N-linked terminal $\alpha$2,3 sialic acids were inhibitory to HA binding (115). It is possible that the factors that affect HA binding to purified CD44 may not be identical to the factors that affect HA binding to CD44 on the cell membrane. In fact Skelton et al. have found that N-acetyl galactosamines (GalNac) on O-linked oligosaccharides only exert their regulatory influence in the context of the cell surface and not on soluble CD44-Ig fusion proteins (115).
6.7 Regulation of HA binding to CD44 by CS and glycosylation

From these data it is proposed that the presence of CS has a positive effect on HA binding and that the presence of fully processed carbohydrates can have a negative effect. Data from the sog8 cells indicate that CS is not essential for IRAWB 14-inducible HA binding, suggesting that one function of CS may be to overcome the negative effects of certain oligosaccharide moieties. One possible explanation is that the small percentage of CS-modified CD44 may act as a nucleus or scaffold upon which other CD44 molecules can bind to create aggregates, which then allow inducible HA binding. For example, it has been demonstrated that the CD44R2 isoform containing the alternatively spliced exon v10 potentiates recognition of chondroitin sulfate moieties present on CD44H and CD44R1 (81). CD44 has been shown to bind to other CS-modified ligands: serglycin (77), the invariant chain of MHC Class II (78) and the aggregation of CD44 can enhance HA binding (15, 55, 108, 123). Alternatively, perhaps only CS-modified CD44 can bind HA in L cells whereas the underglycosylated, 70 kD form of CD44 binds HA in sog8 cells. Regardless of the precise mechanism of this regulation, the ability of CD44 to bind HA is clearly affected by CS addition. Thus, when CS is removed, CD44 can no longer be induced to bind HA by the mAb IRAWB 14. In the absence of both cell surface proteoglycans and certain oligosaccharide moieties however, the binding of HA to CD44 becomes possible.

Inhibitors of glycosylation have previously been reported to affect the HA binding ability of CD44 (110-112, 115-117, 126). This study demonstrates that the presence of CS can also affect the HA binding ability of CD44 in L cells and that this effect depends on the glycosylation state of the cell. Thus, the overall HA binding state of CD44 in L cells appears to be determined by the combined effects of CS synthesis and glycoprotein processing. It is known that cells can vary oligosaccharide and GAG addition depending on their activation state, and changes in the glycosylation of CD44 have been reported to occur in activated macrophages and B cells (126, 128). It will thus be of interest to determine if changes in GAG and oligosaccharide addition occur under physiological conditions to regulate the HA binding ability of CD44. Changes in these post-translational modifications would have to occur on newly synthesized CD44 molecules. In fact, it
was reported that CD44 levels are upregulated after PMA stimulation of T cells and that this
treatment results in the induction of HA binding (57, 63, 101).

6.8 TNFα induction of CD44-mediated leukocyte adhesion by sulfation

Treatment of the SR91 progenitor cell line with the pro-inflammatory cytokine, TNFα,
resulted in increased expression of the cell adhesion molecules ICAM-1 and CD44. Significantly,
TNFα also induced CD44-mediated HA binding by SR91 cells, while IFNγ, another cytokine
involved in inflammatory responses, could not induce HA binding in these cells (Fig. 5.3). The
effects of TNFα on CD44 expression and induction of HA binding required protein synthesis since
cycloheximide, a protein synthesis inhibitor, prevented these effects (214). The observed
induction of ICAM-1 expression by both TNFα and IFNγ was in stark constrast to the inability of
IFNγ to induce HA binding, demonstrating the selective action of TNFα on the increased
expression of CD44 and induction of HA binding mediated by CD44. The ability of IFNγ to
induce ICAM-1 expression in SR91 cells argues against differential expression of IFNγ and TNFα
receptors on the cell surface. Instead, it is more likely that the signal transduction pathways
activated by both the TNFα and IFNγ receptor contain factors in common resulting in the induction
of ICAM-1 expression. However, TNFα must also activate a different set of intracellular signaling
molecules and transcription factors that result in increased CD44 expression and induction of HA
binding that was not observed with IFNγ treatment. TNFα activates a signaling pathway
culminating in activation of NFκB while in contrast, IFNγ activates the JAK/STAT pathway. It
has been reported that stimulation of B cells through the B cell receptor and endothelial cells with
IL-1α induces expression of the egr-1 transcription factor which is responsible for observed
increases in CD44 expression (261, 262). The expression and activity of egr-1 upon TNFα
stimulation was not investigated and does not preclude the involvement of other transcription
factors in increased CD44 expression and induction of HA binding.

In addition to inducing HA binding, TNFα also induced the sulfation of the 85 kD standard
form of CD44 (CD44H). This was a TNFα specific mechanism since the sulfation of CD44 was
not stimulated by IFNγ (Fig. 5.5). Sulfation of CD44 was required for induced adhesion to HA as demonstrated by the abrogation of FL-HA binding in the presence of sodium chlorate during the TNFα stimulation period (Fig. 5.6). The effects of sodium chlorate can be ascribed to inhibition of the synthesis of the sulfate donor, adenosine 3’ phosphate 5’ phosphosulfate (PAPS), and not to induction of cell toxicity as evidenced by cell viability assays and the observed increase in CD44 expression in the presence of sodium chlorate. Data from the sodium chlorate treatments clearly demonstrate that the TNFα-induced increase in CD44 expression alone is not responsible for the induction of HA binding since CD44 expression was increased upon treatment with TNFα and chlorate while HA binding was inhibited. Thus, TNFα can stimulate the sulfation of newly synthesized CD44 molecules, converting CD44 on the cell surface from an inactive to an active, HA binding form of CD44.

TNFα stimulation of SR91 cells induced adhesion to the murine endothelial cell monolayer, SVEC4-10 (Fig. 5.8). The interaction between SR91 leukocytes and the SVEC4-10 endothelial cells was mediated by CD44 on both cells types, as demonstrated by blocking studies with anti-CD44 mAbs (Fig. 5.9). This interaction did not occur with unstimulated or IFNγ stimulated SR91 cells, therefore, this interaction is not dependent upon the upregulation of ICAM-1 expression which was confirmed by the use of anti-ICAM-1 mAbs. The β1 integrin very late antigens, VLA-4 and VLA-5 are involved in leukocyte-endothelial cell interactions and β1 integrins are activated on SR91 cells by TNFα. However, it is unlikely that these cell adhesion molecules are involved in the interaction between SR91 and SVEC4-10 cells as SVEC4-10 cells do not express VCAM-1 (215), the counter-receptor for VLA-4 and VLA-5. The ability of hyaluronidase to inhibit the leukocyte-endothelial cell interaction suggests that HA may play a role in bridging the CD44-CD44 interaction. However, the addition of exogenous HA did not inhibit the interaction and even enhanced it in some experiments. This observation is in agreement with Chiu et al. where cells transfected with the CD44H, CD44R1 and CD44R2 isoforms of CD44 were able to form CD44-mediated aggregates. Aggregation was enhanced in the presence of exogenous HA but inhibited upon hyaluronidase treatment (81). To explain this apparently contradictory data, the SVEC4-10
cells secrete HA (33) which may then be able to bind back onto CD44 on the endothelial cell surface to act as a bridge and promote cellular adhesion between CD44 molecules expressed on the surface of the interacting cells. SVEC4-10 cells are able to bind HA constitutively and, as has been observed in epidermal keratinocytes, a large proportion of HA is bound to CD44 on the cell surface (258). In support of this model which would require CD44 on SR91 cells to bind HA immobilized by CD44 on the SVEC4-10 endothelial cell surface, only TNFα stimulated SR91 cells expressing HA binding CD44 interact with the monolayer. Furthermore, sulfation is required for the interaction between SR91 cells and the SVEC4-10 endothelial monolayer, as demonstrated by treatment of the SR91 cells with the sulfation inhibitor, sodium chlorate (Fig. 5.10). Taken together, TNFα stimulates the sulfation of CD44, converting it from an inactive to an active cell adhesion molecule that can bind the extracellular matrix component HA and thus mediate leukocyte-endothelial cell interactions.

6.9 Sulfation as a mediator of leukocyte-endothelial cell interactions

Sulfation is an additional post-translational modification that can regulate the adhesive function of CD44. It may provide one mechanism for regulating cell adhesion to vascular endothelial cells and may occur on tumor cells to facilitate their extravasation and metastasis. Keratan, heparan, chondroitin, carbohydrate and tyrosine sulfate have been detected on alternatively spliced, higher molecular weight forms (116-200kD) of CD44 (37, 109, 263, 264), however, the specific role of sulfation itself is not clear. In TNFα stimulated SR91 cells, the 85 kD form of CD44 was the major sulfated moiety. However, this does not preclude the possibility that expression of larger isoforms of CD44 were induced by TNFα. CD44 isoforms containing the variant exon v10 may have been induced by TNFα as demonstrated by reactivity with the anti-CD44v10 mAb, 2G1. However, these v10 containing isoforms may be expressed in such small quantities that they are below the level of detection by western blot and [35S]sulfate labeling. Alternatively, this CD44 isoform may not be sulfated and therefore not detected by [35S]sulfate labeling. However, if the CD44v10 isoform is not sulfated, then it cannot be responsible for
TNFα induced HA binding and leukocyte adhesion mediated by CD44 which are dependent on sulfation. Investigation of the CD44 isoforms expressed in SR91 cells and induced by TNFα can be achieved by reverse transcriptase polymerase chain reaction (RT-PCR) which is now being pursued by others in the laboratory.

Data from sulfo-amino acid analysis provided no evidence for the tyrosine sulfation of CD44 (Fig. 5.15), whereas chemical or enzymatic removal of carbohydrate provided preliminary evidence for carbohydrate sulfation (Fig. 5.13). Specifically, there was indirect evidence that TNFα inducible sulfation of CD44 occurred on O-linked oligosaccharides. However, the precise identification of the sulfated residues and their location within CD44 remain to be determined.

The selectin ligands are cell-adhesion molecules involved in leukocyte-endothelial cell interactions that are also sulfated (reviewed in 132). These molecules mediate the initial rolling interactions between leukocytes and high walled endothelial venules (HEV) (3, 132, 265). Lymph node HEV incorporate large amounts of sulfate and constitutively express sulfated glycoproteins on the cell surface (266). Carbohydrate sulfation of two selectin ligands, GlyCAM-1 and CD34, occurs in HEV on O-linked glycans and is required for high affinity binding to L-selectin (135, 136), as treatment with chlorate abolishes binding. Tyrosine sulfation of the P-selectin ligand, PSGL-1, present on leukocytes, together with recognition of the sialyl LewisX-like epitope is important for high affinity binding to P-selectin (130, 131, 137). Thus the sulfation of cell adhesion molecules on HEV and leukocytes may occur to facilitate leukocyte-endothelial cell interactions. The induction of CD44 sulfation by TNFα provides one potential mechanism for regulating leukocyte adhesion during an inflammatory response.

The interaction between CD44 and HA can be compared to that of the selectins and their ligands. The N-terminal regions of CD44 and the selectins are likely to be related as the CD44 Link module is structurally similar to C-type lectin domains (48) which are expressed by selectins. Both CD44 and the selectins utilize this C-type lectin domain to bind carbohydrate ligands and interaction with their ligands can mediate leukocyte-endothelial cell interactions and leukocyte rolling under flow conditions (45, 48, 267). From work presented here and elsewhere, it is
evident that sulfation can regulate HA binding by CD44 and it is also required for high affinity
selectin binding. However, it should be noted that L- and P-selectin bind to sulfated ligands
whereas sulfation occurs on the CD44 lectin molecule itself which then influences its interaction
with a non-sulfated ligand, HA. Whether the C-type lectin-like region of CD44 can bind sulfated
moieties on itself to induce HA binding by aggregating the HA binding domain or causing a
conformational change in the lectin binding domain remains to be determined.

6.10 Mechanism of TNFα induced sulfation

Given that TNFα was able to induce the sulfation of CD44, thereby activating its adhesive
function, very little is known about the enzymes responsible for the sulfation of CD44 and how
TNFα regulates them. Signals transduced by TNFα receptors may induce expression of a
sulfotransferase and/or activate its enzymatic activity. Several sulfotransferases have been cloned
but the majority are cytosolic enzymes involved in sulfation of hormones or the modification of
tyrosines with sulfate. Recently, carbohydrate specific sulfotransferases have been cloned which
specifically add sulfate on Gal and GlcNac in the carbon 6 position (144). Both of these
sulfotransferases act on the L-selectin ligands GlyCAM-1 and CD34 which are expressed on HEV.
As noted previously, sulfation of GlyCAM-1 and CD34 on sialyl Lewis^x carbohydrates is required
for L-selectin recognition. While the Gal-6-sulfotransferase is widely expressed, the GlcNac-6-
sulfotransferase appears to be restricted to HEVs. However, it is not known if expression of this
GlcNac-6-sulfotransferase, or for that matter other carbohydrate-specific sulfotransferases, is
induced by the action of TNFα or other pro-inflammatory mediators on leukocytes or endothelium
during an inflammatory response. From these reports and data implicating O-linked carbohydrates
on CD44 as the target of sulfotransferases, an attractive hypothesis is that in SR91 cells, TNFα
induces the sulfation of CD44 on O-linked oligosaccharides, potentially on GlcNac moieties by the
activation of a GlcNac-6-sulfotransferase. In support of this hypothesis, L-selectin ligands
expressed on HEV contain GlcNac-6-sulfate in the context of core 2 O-linked oligosaccharides
(135, 136), which has been observed in only a few examples beyond the L-selectin ligands. Core
2 oligosaccharide biosynthesis appears to be important for selectin ligand recognition during inflammation (268). Although the activity of core 2 β1-6 N-acetyl glucosaminytransferase (C2GnT) was not assayed in TNFα stimulated SR91 cells, it is possible that TNFα can influence core 2 oligosaccharide addition by stimulating the activity of C2GnT similar to T cell activation increasing C2GnT activity (269). Thus a situation where TNFα activates C2GnT and sulfotransferase activity could be envisaged, resulting in CD44 sulfation on O-linked oligosaccharides and induction of HA binding ability.

6.11 Role of sulfation in regulating cell adhesion in other systems

It is notable that sulfated glycoconjugates have been found in several species such as bacteria, brown algae, protozoans, marine sponges, echinoderms, and mammals. In all these cases, sulfation has been implicated in regulating cellular interactions and adhesion (reviewed in 129). Phytohemagglutinin (PHA) stimulated peripheral blood lymphocytes (PBLs) expressed sulfated CD45 and CD43 and sulfation was implicated in the modulation of homotypic aggregation induced by anti-CD43 mAbs (270). Recently, tyrosine sulfation of the chemokine receptor, CCR5 was reported and contributed to the binding of chemokines MIP-1α and β and to the binding and entry of HIV-1 (271). Another chemokine receptor involved in HIV-1 entry, CXCR4 is also sulfated and sulfated proteoglycans can facilitate the attachment of other enveloped viruses such as HSV-1 (272). Thus, sulfation may occur on leukocyte cell surface proteins to regulate cell, viral, and chemokine interactions.

6.12 Characterization of HA binding by SR91 cells in response to other soluble factors

The effect of various other soluble factors involved in inflammatory responses on the HA binding ability of SR91 cells was examined and it was determined that neither bacterial lipopolysaccharide (LPS), nor the cytokine IL-1β, induced CD44-mediated HA binding (Fig. 5.4). This result may be explained by a lack of expression of the receptors for LPS and IL-1β on SR91
cells, CD14 and the IL-1 receptor, respectively. Similarly, the chemokines, MCP-1 and MIP-1α were also unable to induce HA binding in SR91 cells (Fig. 5.4), potentially because SR91 cells also did not express the appropriate receptors. Chemokines are chemotactic cytokines which have a critical role in attracting different types of leukocytes to sites of inflammation (reviewed in 273). These small molecules can also regulate adhesion molecule expression and activation in leukocytes. Of the growing number of chemokines, the subfamily of CC chemokines contain a number of members which are considered to be pro-inflammatory. Both MCP-1 and MIP-1α fall in this category and were chosen for these studies because they specifically target their actions to monocytes. However, it is possible that they did not have an effect on HA binding by SR91 cells because SR91 cells retain the characteristics of myeloid progenitors and not mature monocytes and thus were unable to respond.

While the SR91 cells have proved to be a very good model system to study the effects of TNFα stimulation on CD44 adhesive function, several caveats regarding these cells must be stated. One question that emerged out of these studies was whether the effects observed upon TNFα treatment of SR91 cells were related to the myeloid or progenitor nature of these cells. Secondly, because these cells were derived from a patient with acute lymphocytic leukemia (193), it is possible that the observed HA binding induced by TNFα is a characteristic of all cancerous cells whether they are of myeloid or lymphoid origin. Thirdly, the SR91 cell line was derived from a lymphocytic leukemia, which, after some time in culture, differentiated into a myeloid progenitor cell and thus its responses to soluble factors may not have been representative of normal ex vivo blood cells. Finally, it was important to establish whether CD44 sulfation and induction of HA binding occurred in other cell types and normal cells. Thus, these caveats were addressed in the following ways. In preliminary studies not reported here, PBMCs from 8 different patients with various different stages of myeloid leukemia were tested for TNFα induced HA binding. Two patient samples did express a very small population of cells which could be induced to bind HA after TNFα treatment. In one case HA binding was partially dependent on sulfation and in the other case, not dependent on sulfation (data not shown). TNFα inducible HA binding was only
observed in patient samples containing a CD14 positive population comprising more than 60% of cells which were selected on the basis of their larger size and increased granularity. Considering that 6 out of 8 leukemic samples did not exhibit TNFα induced HA binding, it is more attractive to think that monocytes are more likely to respond to TNFα by binding HA, not cancerous cells.

Differentiation along the myeloid lineage is regulated by various cytokines in the bone marrow. The earliest myeloid cells have been identified by characteristic expression of cell surface markers and these cells are CD33<sup>b</sup>, CD15<sup>-</sup> (sLe<sup>x</sup>), CD44<sup>hi</sup> (274). This subpopulation of cells do not express CD11b (Mac-1), CD14, or any lymphoid markers. Commitment to monocyte differentiation is suggested by the acquisition of an LFA-1<sup>hi</sup>, CD11c<sup>+</sup> subset and the upregulation of CD33 on the cell surface. In later stages of myelopoiesis, cells become CD11b<sup>+</sup>, CD14<sup>+</sup> and lose CD33 expression, becoming mature monocytes. A murine CD34 positive progenitor cell line, B6sutA, was examined to determine if progenitor cells could be induced to bind HA (data not shown). TNFα did not induce HA binding in these cells, nor for that matter did cytokines which induce myelopoiesis, such as granulocyte-macrophage stimulating factor (GM-CSF), granulocyte stimulating factor (G-CSF), macrophage stimulating factor (M-CSF), or stem cell factor (SCF). Again, this observation may be explained by the fact that B6sutA cells are a transformed cell line that might have lost its ability to respond to these factors. While it has been reported using the human CD34 positive myeloid progenitor cell line, KG1a, that approximately 20-25% of these cells constitutively bind HA in a CD44 dependent manner (219), TNFα had no effect on the HA binding ability of these cells (data not shown). Isolation of CD34 positive haematopoietic progenitor cells from bone marrow revealed that these cells expressed abundant surface CD44 but only 13% of cells spontaneously adhered to HA (99). Short-term culture on methylcellulose showed that these HA binding CD34 positive cells comprised primarily granulo-monocytic (CFU-GM; 20%) and erythroid (BFU-E; 7%) cells. This adhesion to HA could be enhanced by PMA, treatment with anti-CD44 mAbs, or cytokines such as GM-CSF, IL-3, and SCF, however, it is not known if TNFα could also enhance HA binding by these cells. Potentially, TNFα exerts its effects on more mature cells of the myeloid lineage such as SR91 cells and monocytes whereas HA
binding is induced in CD34 positive early progenitor cells by cytokines involved in the growth and differentiation of myeloid cells. In support of this, it was found that CD34 positive progenitor cell lines such as TF1 and MO7 are induced to bind HA after treatment with GM-CSF, IL-3, or SCF (275).

6.13 Induction of HA binding on PBMCs by soluble factors

TNFα induced HA binding in large, granular PBMCs after 72 hours (Fig. 5.17) and was mediated by CD44 (K. Brown, unpublished data), in agreement with reported observations (30). Treatment of PBMCs with IL-1β had no effect on HA binding (Fig. 5.17), in contrast to data reported by Levesque and Haynes where IL-1α and β were both able to induce CD44-mediated HA binding on CD14 positive PBMCs (30). Based on the inability of IFNγ to activate HA binding on SR91 cells, PBMCs were not stimulated with this cytokine. However, contradictory results have emerged using PBMCs, where one group found IFNγ activated the ability of monocytes to bind HA (276), whereas others did not (30). In addition, the pro-inflammatory CC chemokines, MCP-1 and MIP-1α activated HA binding in a subpopulation of PBMCs (Fig. 5.17). By contrast, others failed to observe an induction of HA binding after MIP-1α treatment of PBMCs; MCP-1 was not tested in those experiments (30). Preliminary data from my studies also indicate that LPS activates the adhesive function of CD44 by inducing HA binding, as observed by others (30, 276). However, the LPS experiment has not been repeated enough times to make any firm conclusions and the contradictory data presented here highlights a significant caveat to data from PBMC studies; caution must be exercised when making conclusions from PBMCs due to the variability in donor responses to various soluble factors.

Over a number of donors, TNFα induced HA binding was usually observed in a subpopulation of PBMCs. This subpopulation was defined by virtue of its large size (FSC) and greater granularity (SSC) by flow cytometry (Fig. 5.18), consistent with the presence of monocytes and granulocytes in this population. The smaller, less granular cells did not bind HA and could not be induced to bind HA by any of the soluble factors tested. These cells probably
represent the PBL population and do not bind HA in response to TNFα (data not shown), LPS, or IFNγ (276). Closer examination of the size-selected population of PBMCs revealed that the CD14 positive cells in this group inducibly bound HA in response to pro-inflammatory factors (Fig. 5.19). By purifying monocytes on the basis of adherence to plastic, it was demonstrated that purified monocytes responded to TNFα by binding to HA in a similar manner to unseparated PBMCs (Fig. 5.20) and gating on CD14 positive cells revealed that they had similar HA binding profiles to the size selected cells (Fig. 5.21). However, not all CD14 positive cells purified by adherence to plastic bound HA; generally only a proportion of cells responded to the TNFα treatments by binding HA. This phenomenon has been observed by others using freshly explanted cells and other soluble factors and may reflect the heterogeneity of ex vivo cells from different donor populations (28, 277). Increased HA binding by monocytes was observed after TNFα treatment in combination with incubation with a CD44 activating mAb (127). Given the data, all indications are that monocytes are one of the probable targets of TNFα, resulting in the induction of HA binding. While it cannot be said that all the HA binding cells are CD14 positive, mature monocytes, or even that all CD14 positive cells bind HA, is possible that a mixed population of myeloid cells can be induced to bind HA by TNFα.

It is apparent that many of the soluble pro-inflammatory factors tested in my studies have specific effects on monocytes, specifically the induction of HA binding (here and 30). While there are some discrepancies using certain cytokines such as IL-1β, the ability of TNFα to induce HA binding by monocytes in PBMC suspensions is evident. TNFα may play an important role in the formation of cytokine networks to integrate leukocyte adhesion at inflammatory sites. It has been reported that treatment of PBMCs with IL-2 or IL-15 induces HA binding by monocytes (30). This binding however, was blocked by a TNFα neutralizing antibody. The ability of IL-2 and IL-15 to induce HA binding by CD44 on monocytes in PBMC suspensions was likely mediated by cytokine effects on PBLs, with the resulting production of TNFα by these lymphocytes to induce monocyte CD44 binding to HA. PBLs themselves were not induced to bind HA in the presence of TNFα (my observations), IFNγ, or LPS (276). Finally, T cells activated by antigen,
superantigen, or alloantigen also acquire the ability to bind HA (20, 22, 23, 25-27) and CD44-mediated adhesive functions have been correlated with activated T cells in chronic inflammatory diseases (278). Taken together, TNFα may play a central role in cytokine regulation of the ability of monocyte CD44 to bind HA. This is an important point given the role of TNFα in the pathophysiology of diseases such as toxic shock syndrome, rheumatoid arthritis and inflammatory bowel disease and given the role of activated monocytes as mediators of the inflammatory response.

The action of TNFα was not restricted to the induction of HA binding in PBMCs but also appeared to either upregulate the expression of CD14 on monocytes or actually increase the number of CD14 positive cells after a 72 hour treatment (Fig. 5.19 and 5.20). It is possible that TNFα upregulates CD14 expression on monocytes to prime them for surveillance against bacteria that bear its counter-receptor, LPS. A more attractive model favours TNFα influencing myelopoiesis to stimulate differentiation of CD33 positive cells into CD14 positive mature monocytes. Immature dendritic cells (DCs) derived from peripheral blood monocytes can be stimulated to mature in TNFα (279) and to bind HA in a CD44-dependent manner (31). Thus, TNFα plays a role in differentiation and the activation of adhesion in immune cells. CD44 and CD44-mediated adhesion has been shown to be important in haematopoiesis as well. Blocking the HA binding region of CD44 with the anti-CD44 mAb, KM201, in long term bone marrow culture completely blocked production of lymphoid and myeloid cells (196). More significantly, inactivation of CD44 by gene ablation, resulted in the altered tissue distribution of myeloid progenitors, indicating that myeloid progenitor egress into the blood was defective (24). Given the growing body of evidence, it is appealing to speculate that TNFα and CD44 mediated adhesion act together in myelopoiesis and the mobilization of myeloid cells during inflammatory responses.

In this study and findings by others (30), low level HA binding was observed by PBMCs stimulated with TNFα at 24 and 48 hours (data not shown), peaking at 72 hours. This time course of HA binding is compatible with the time required for an influx of peripheral blood monocytes at inflammatory sites during wound repair (280, 281) and for monocyte extravasation during
delayed-type hypersensitivity responses (282). In addition, it was reported that human serum can induce some HA binding on peripheral blood monocytes in 24 hours (29). Thus, a scenario can be envisaged where early low level HA binding induced by, as yet unidentified serum components, mediates rolling on endothelium and some monocyte egress at 24 hours, initiating the inflammatory response. This would be followed by increasing cytokine-induced HA binding by monocytes over time to augment the inflammatory response. Thus, the increased TNFα-induced HA binding in monocytes at 72 hours is consistent with normal immune function.

6.14 Induction of HA binding by TNFα on PBMCs is regulated by sulfation

Similar to SR91 cells, TNFα-induced HA binding by PBMCs was decreased by approximately 30-80% with simultaneous treatment with chlorate, the sulfation inhibitor. Unlike the situation with SR91 cells, complete abrogation of HA binding was not achieved by treatment of PBMCs with 50 mM sodium chlorate for 72 hours concurrent with TNFα stimulation. HA binding molecules other than CD44 may be expressed on the surface of PBMCs that are not dependent on sulfation for adhesion to HA explaining the observed differences between PBMCs and SR91 cells. Alternatively, the presence of other cells or factors produced by these cells, in the mixed PBMC suspension may counteract the inhibitory effects of sodium chlorate. Evidence for this comes from purified monocytes, where HA binding induced by TNFα was almost completely blocked by the action of sodium chlorate (Fig. 5.20 and 5.21). Sodium chlorate is an analogue of the sulfate donor in PAPS biosynthesis and therefore acts by interfering with PAPS synthesis. It is possible that in the 72 hour treatment period, the concentration of chlorate used was insufficient to totally block PAPS synthesis. It should be noted that PBMCs were cultured at higher cell densities than SR91 cells; culture at lower densities resulted in PBMC death. Alternatively, PAPS synthesis may have been blocked by chlorate, but there may also have been a larger pre-existing pool of PAPS available to PBMCs. Guiacol is another type of sulfation inhibitor, which, as a substrate for sulfation reactions, depletes the intracellular pool of PAPS in cells. It has been shown that the use of different classes of sulfation inhibitors together often results in synergistic
effects and complete inhibition of sulfation (283). Perhaps TNFα induced HA binding by PBMCs can be completely blocked with chlorate in combination with guiacol.

It was not apparent whether sulfation was the mechanism by which the CC chemokines MCP-1 and MIP-1α induced HA binding in PBMCs. The source of the confusion lies in contradictory data obtained from too few donors combined with variability in donor responses. While a decrease in HA binding was observed with chemokine treatment of PBMCs in combination with chlorate, this was only observed one time and the decrease in HA binding was approximately 20-50%. Thus, it remains to be determined whether MCP-1 and MIP-1α induced HA binding is sulfation dependent. Other factors have been shown to influence HA binding activated by different stimuli. Levesque and Haynes demonstrated that TNFα induced HA binding in ex vivo monocytes correlated with a 5-10 kD decrease in \( M_r \) of the standard form of CD44, CD44H, which was attributed to a loss of some post-translational modification (127). In the THP-1 monocytic cell line, activation of HA binding by LPS correlated with an induction of sialidase activity and was blocked by a sialidase inhibitor, suggesting that the removal of sialic acid residues resulted in HA binding (277). Pro-inflammatory cytokines such as TNFα and IFNγ upregulated the expression of CD44 variant isoforms, particularly v6 and v9 in myelomonocytic cell lines (284) and culture of ex vivo peripheral blood monocytes in human serum also upregulated expression of CD44v6 and CD44v9 (29). In the case of the monocytes there was a concomitant activation of HA binding. However, TNFα activation of HA binding by DCs was directly linked to the upregulation of CD44v6, not CD44v9, as anti-CD44v6 mAbs were able to block HA binding (31). These studies suggest that an increase in the expression of CD44 variant isoforms v6 and v9 may be correlated with HA binding and one study goes so far as to demonstrate that CD44v6 is required for HA binding in DCs. However, experiments from SR91 cells do not indicate a reduction in the \( M_r \) of CD44 upon TNFα stimulation. While the relative contribution of terminal sialic acid moieties on SR91 cells or PBMCs was not examined, no gross shift in \( M_r \) of CD44 was observed in SR91 cells which would have been consistent with a loss of sialic acid upon TNFα treatment. Although no higher \( M_r \) forms of CD44 were observed in TNFα stimulated SR91 cells, it is possible that
some CD44 variant isoforms were upregulated that were not detected by western blot. Analysis of mRNA from SR91 cells and peripheral blood monocytes by RT-PCR of CD44 should resolve this issue.

It is tempting to speculate that sulfation is a general mechanism by which cells of the myeloid lineage activate CD44-mediated adhesion. Whether it be CD34 positive early myeloid progenitors, CD33 positive myeloid progenitors, or CD14 positive monocytes, each of these cell types may potentially modify CD44 with sulfate in response to a variety of factors. This was the case with the CD34 positive, early myeloid progenitor cell line KG1a. KG1a cells constitutively bound HA which was reduced significantly by incubation in sodium chlorate. CD44-mediated adhesion is important in many aspects of myeloid function: in myelopoiesis, leukocyte-endothelial interactions, and leukocyte extravasation. Given that sulfation is a post-translational modification that can occur on eukaryotic cell surface molecules to regulate cell adhesion, it is perhaps worthwhile to re-examine the extent and type of sulfation occurring on leukocytes during different biological processes.

6.15 The regulation of CD44-mediated HA binding by sulfation: a model

It has been suggested that IRAWB 14 induces HA binding by CD44 by either promoting aggregation or by altering the conformation of CD44 (15, 123). A similar case can be made for TNFα induced HA binding on SR91 cells. If this is the case, then the presence of sulfated moieties at the cell surface, either CS-modified CD44, sulfated oligosaccharides on CD44, or sulfated CD44v isoforms, may promote aggregation or generate a favourable HA binding conformation of CD44 while the presence of other terminal oligosaccharide moieties may have the opposite effect. To help explain how both sulfate and carbohydrate may regulate the HA binding ability of CD44, two models have been proposed (Fig. 6.1). For both models, sulfated CD44 can refer to CS-modified CD44 in L cells, CD44 inducibly sulfated on carbohydrates by TNFα on SR91 cells, or CD44 isoforms modified by GAGs (91, 104) or sulfo-tyrosines (264) on the additional peptide sequences inserted in the protein backbone. The aggregation model proposes
Figure 6.1. Models to explain the induction of CD44-mediated HA binding. The aggregation model (A) and the conformational model (B) are depicted. The non-binding and HA binding states are as shown. The inclusion of additional sequence present in the CD44\textit{v} isoforms is indicated by the black box, (\textbullet) represents potential N-linked oligosaccharides, (\textbullet\textbullet) potential O-linked oligosaccharides, (\textbullet\textbullet\textbullet\textbullet) GAG sidechains, and (\textastertisk) sulfate moieties.
that sulfated CD44 acts as a nucleus or scaffold to which other CD44 molecules can bind. This binding occurs via sulfated side chains and promotes the aggregation or clustering of non-sulfated CD44H molecules. Such clustering can then facilitate HA binding by a population of cells or further aggregation induced by IRAWB 14 resulting in increased HA binding. In the absence of sulfate on CD44, certain oligosaccharide moieties prevent aggregation, and although IRAWB 14 can bind to CD44, it cannot cluster sufficient CD44 molecules to allow HA binding. In the absence of both CS and certain oligosaccharide moieties (as is the case on sog8 cells), CD44 can form tighter aggregates as a result of the loss of charged carbohydrates and GAGs, leading to some constitutive HA binding and additional IRAWB 14 inducible binding. Notably, the aggregation state of CD44 has been correlated with its HA binding ability (55, 108).

The conformational model proposes that sulfate modification of CD44 can cause a conformational change in the molecule such that SR91 cells can bind HA, and in L cells, stabilize the HA binding conformation induced by addition of the IRAWB 14 mAb (Fig. 6.1). Potentially this may occur through interactions between sulfated moieties and negative regulatory carbohydrates on CD44. As with the aggregation model, one CS modified CD44 molecule can stabilize the conformation of several CD44 molecules, thus explaining why CS modified CD44 is not present in equimolar amounts to CD44H. Upon the loss of the CS modified CD44, the HA binding conformation can no longer be stabilized and the presence of bulky carbohydrate moieties prevent IRAWB 14 from inducing the HA binding conformation. However, when sufficient carbohydrates are removed, the HA binding region of CD44 is much less restricted and both constitutive and inducible binding conformations are possible (Fig. 6.1). In contrast to L cells, it was not clear whether all CD44 molecules on the surface of SR91 cells were sulfated, although it appears likely that all newly synthesized CD44 contained sulfated oligosaccharides. Thus it is not clear whether non-sulfated CD44 was involved in HA binding. It is more likely that only CD44 molecules that were inducibly sulfated by the action of TNFα could bind HA, explaining the heterogeneous profiles of HA binding by flow cytometry. While both of these models are not necessarily mutually exclusive, they do provide the basis for further predictions which can now be
tested experimentally in the L cell and SR91 cell systems either by transfection of CD44 constructs that contain more sites for addition of sulfate or by the overexpression of enzymes that regulate the glycosylation and sulfation state of CD44.
CHAPTER 7

References


